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THE ZYMOPHAGE [1]

by

CARL C. LINDEGREN and YONG NYU BANG

(Received October 9, 1960).

INTRODUCTION.

This paper reports experiments demonstrating that an intracellular parasite, the zymophage, can infect the protoplasm of *Saccharomyces*. The existence of the zymophage was first inferred in 1954, when Dr. LINDEGREN observed plaques on agar-plates on which cells of culture 13777 of the Lindegren Breeding Stock [2]) of *Saccharomyces* had been spread; a formal announcement was made in 1958 at a meeting of the American Society of Brewing Chemists in Detroit. Plaques sometimes appear in the film of haploid yeast cells growing on an agar plate; certain cultures seem more likely than others to produce them. Plaques appear at certain times on many cultures (as if a general infection were occurring) followed by long periods during which no plaques are observed. More than 40 haploid cultures were observed to produce plaques during 1957. Many of the plates, on which the plaques appeared, had been used to study the effects of various poisons (applied by saturating paper discs). Examination of such plates often reveals many plaques on some plates (fig. 1), while other plates show none. Plaques were also found on plates to which no poison had been added, proving that the poison was not the cause.

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[1]) This work has been aided by a grant from the American Cancer Society.

[2]) The cultures of the Breeding Stock cannot be given a species designation, since they are the offspring of a series of hybrids developed by interbreeding the following five species: *Saccharomyces microellipsoideus*, *S. cerevisiae*, *S. carlsbergensis*, *S. chodati* and *S. kluyveri*.

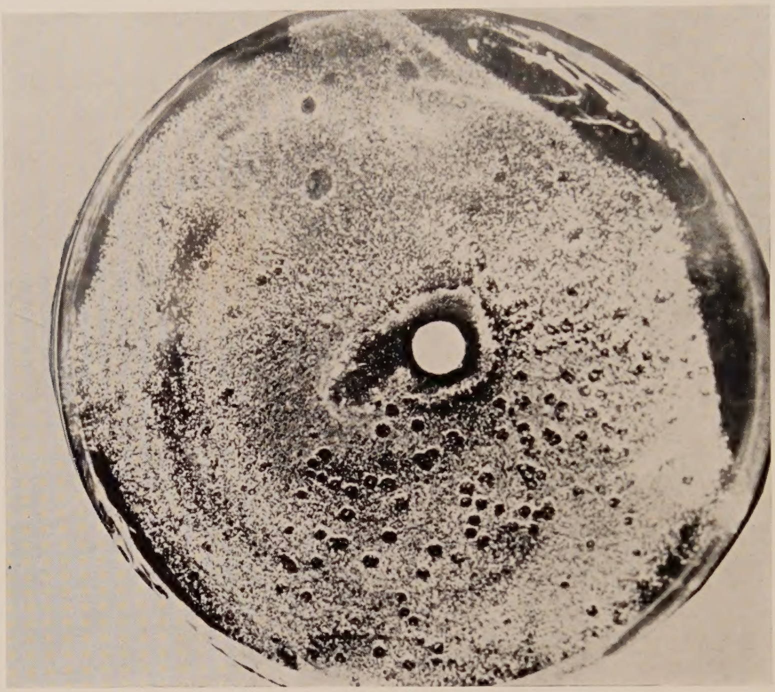

Fig. 1. Culture 22031 growing on an agar petri plate on which plaques appeared "spontaneously". The white disc in the center contained a poison which was being tested against this culture. × 1.

EXPERIMENTAL.

The Propagation of Plaques from Plaques.

When culture 13777 (which is incapable of utilizing sucrose) was spread on agar (in the preliminary observations of 1954), a thin film of cells appeared on the surface of sucrose-nutrient-agar plates, presumably nourished by the nutrients obtained from the peptone and liquid yeast extract in the medium. Many sucrose plates showed 20 or more plaques. The problem of propagating the plaques was first assigned to SANTOS who applied some of the conventional methods of bacteriophage propagation without success and reported his results in a masters' thesis (1954). It is possible that the very thin film of cells which appeared on the sucrose-agar (seeded with the culture which could not use sucrose) was a major factor in the first appearance of plaques, since a thicker film might have rapidly overgrown the plaque area.

The phenomenon of plaque formation due to virus infection is sometimes partially obscured by the appearance of "plaques" arising from other causes. "Water droplet plaques" often appear on plates after water vapor has condensed on the surface of the agar. When a water droplet condenses on the agar surface of an inverted plate, it dries to leave a clear circular area which is visible when the plate is inspected by holding it up to the light. When yeast cells are spread over a plate on which water droplets have condensed and subsequently dried, they do not grow completely over the surface of the area occupied by the drop but leave an open space in this area. "Plaques" arising in this manner are soon overgrown. Confusion of "water droplet plaques" with viral plaques can be avoided if plates used for the propagation of zymophage are carefully inspected before smearing them with yeast cells and the droplet "scars" marked with a pencil.

Plaques were occasionally reproduced by Miss BANG by the following procedure: Material scraped from the plaque with a flat platinum wire was inoculated into 4.5 ml nutrient broth and incubated 15—18 hours at 30° C. After incubation, the culture tube was shaken gently to suspend the cells and 8 drops of the broth-suspension were transferred to an agar-plate with a dropping pipette and the plate tilted from side to side to distribute the suspension over the surface of the agar. The plate was covered immediately with an unglazed porcelain cover to remove excess moisture and was not moved for 4 hours. The cells were allowed to grow at room temperature, approximately 29° C. Plaques were detected from 4 to 12 hours after inoculation.

Fig. 2 shows a photograph of a yeast film produced by this procedure, containing clearly defined plaques.

The following standard formula was used for both agar and broth:

agar	30.0 g
glucose	5.0 g
peptone	3.5 g
KH_2PO_4	2.0 g
$MgSO_4$	1.0 g
$(NH_4)_2SO_4$	1.0 g
yeast extract	2.0 g
vitamins	1.0 ml (vitamin mixture solution)
dist. H_2O	1000.0 ml
pH	5.6 — 6.0

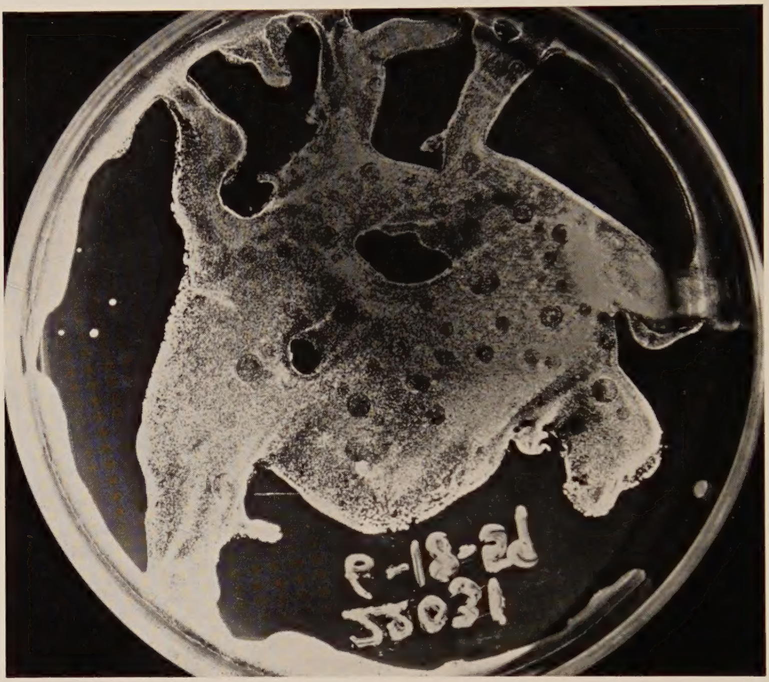

Fig. 2. Plaques appearing in the film of cells of culture 22031 obtained by incubating a plaque from the original plate (fig. 1) in broth and spreading eight drops from the broth onto an agar plate. × 1.

The following experiment was performed using plaques which had been propagated from plaques appearing on culture 22077: 12 plaque areas were scraped, as indicated above. The scraped material was incubated in 4.5 ml broth for 18 hours and 0.5 ml of the suspension was spread over the surfaces of each of 12 separate petri dishes. As a control, 12 areas in which plaques were not visible were scraped and incubated in broth which was then spread over agar on separate dishes. Table 1 shows the numbers of plaques which appeared on the different plates. These data indicate that plaques appear in films of yeast growth derived from plaque areas more readily than in films of growth from cells which were transferred from control areas which did not contain plaques. This experiment was not always successful and plates containing plaques were not always available; other means were developed to detect the presence of a viral infection in the cultures.

TABLE 1.
A comparison of the number of plaques produced by yeast cultures developed from "plaque" and "normal" areas.

Replication	Plaques developing in culture from	
	Normal Area	Plaque Area
1	0	35
2	0	37
3	1	22
4	1	22
5	4	16
6	0	28
7	4	3
8	0	17
9	0	12
10	2	10
11	0	1
12	0	3

The Moist Chamber Slide.

Since plaque formation involves the lysis of cells, this phenomenon was studied under high magnification by use of a specially constructed moist chamber slide. The slide was made of aluminium, one inch wide and three inches long and a little less than one mm thick. A hole 13 × 37 mm was punched in the center of the slide. It was covered on the bottom by a No. 2 cover glass 20 × 50 mm sealed in place with stopcock grease. A No. 1, 20 × 50 mm cover glass was covered with a thin layer of agar by allowing agar to flow over the surface of the cover glass. As soon as the agar was solid the edges were trimmed, leaving an agar rectangle about 10 × 30 mm. The edges of the upper opening of the aluminium slide were smeared with stopcock grease. The agar was inoculated with yeast and the cover glass pressed into the stopcock grease. This prepared a moist chamber sufficiently thin so that a high power objective could focus upon the cells growing on the agar and a condenser of high resolving power could be used to illuminate the cells. Either phase or dark field can be used to study the preparation.

Cells scraped from a plaque (or from a culture known to be infected) were inoculated into broth and allowed to grow for 24 hours, and the broth cultures provided the inocula for the moist chamber slides. Colonies grown on the moist chamber slide were observed in the process of disintegration, leaving no doubt that the debris on the agar had arisen from previously viable cells. In some instances it looked as if single cells had extruded a long string of cellular debris (fig. 3).

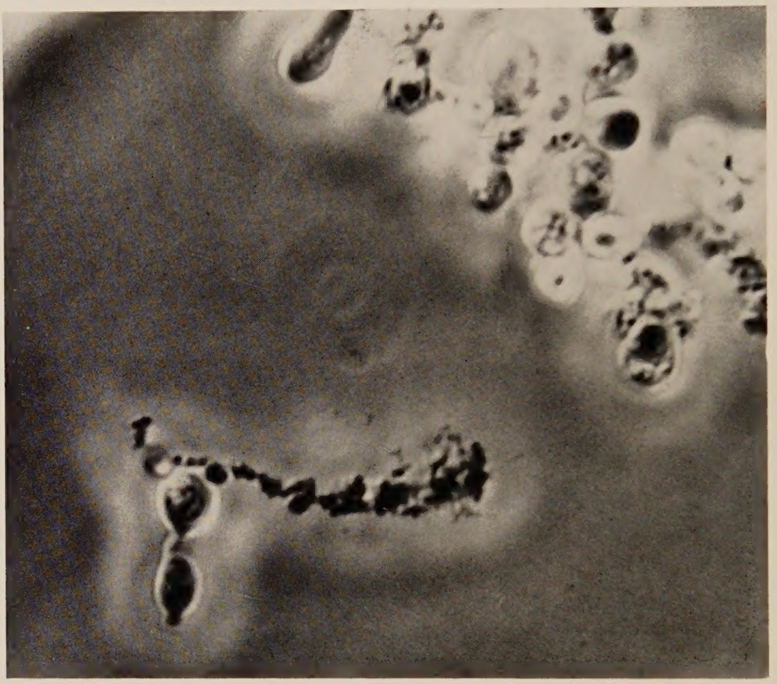

Fig. 3. A cell from which debris has been extruded in a manner suggesting explosive expulsion of the protoplast. × 2500.

Agglutinated Debris.

It was observed on many occasions (when suspensions of cells from a subculture were examined with a microscope) that the colonies (the cells of which had disintegrated) formed a single agglutinated mass (fig. 4). Small microcolonies in which the cells were all agglutinated, as is characteristic of haploid yeast, were found associated with agglutinated masses of debris in which no intact cells were viable, showing that the debris had been derived from the cell clusters (fig. 5).

The Overflow Technique.

An interesting observation was made in an attempt to isolate and

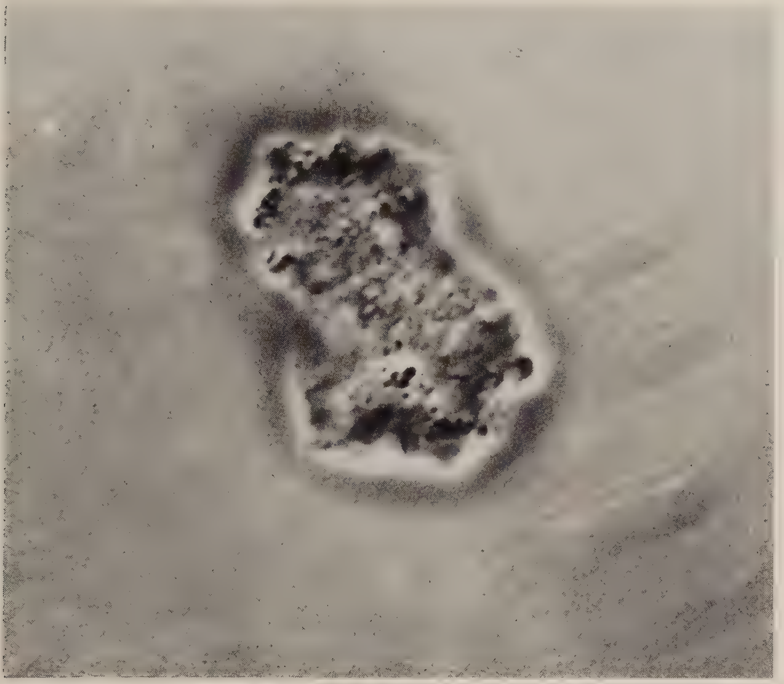

Fig. 4. An agglutinated mass of cellular debris. × 2000.

identify susceptible clones. Half of the surface of an agar plate was smeared with "background" cells from a culture known to have produced plaques, and separate drops of suspensions of cells from a variety of other cultures were placed on the clear side of the plate. The plate was then tilted and the suspensions allowed to flow over the smeared surface. The "background" cells need every advantage in growth if they are not to be washed off. To minimize the movement of the background cells during the overflow process, the cells were allowed to grow for 12 hours and the agar surface was partially dried by placing an unglazed porcelain cover over the petri dish for a short time before the overflow occurred. When these precautions are taken there is little movement of the background cells when a drop of water without cells or virus is allowed to run over the plate as a control test. In some overflow streaks complete clearing of the background cells occurs; in others, long slender streaks of uniform width occasionally broken by intervening regions appear on the background (fig. 6). The long slender, "viral" streaks may

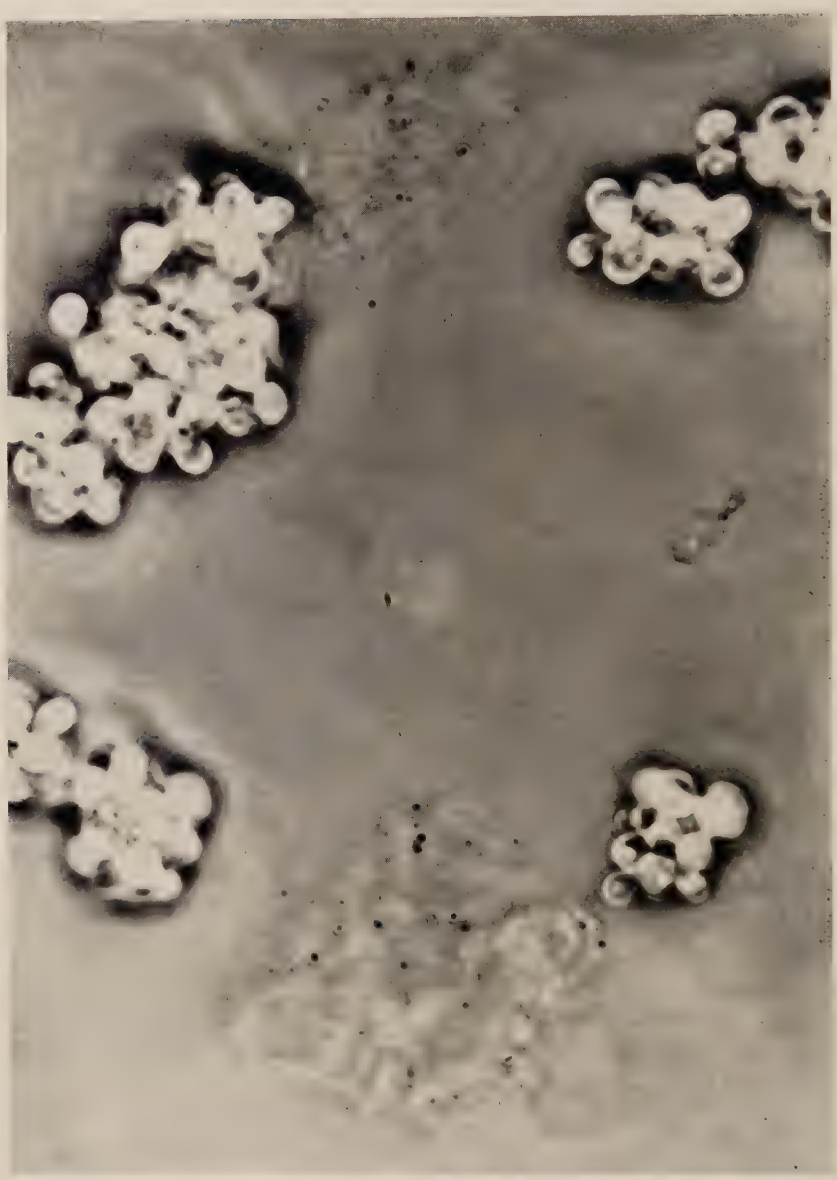

Fig. 5. Agglutinated masses of cellular debris associated with agglutinated cells photographed with phase contrast in the moist chamber. × 2000.

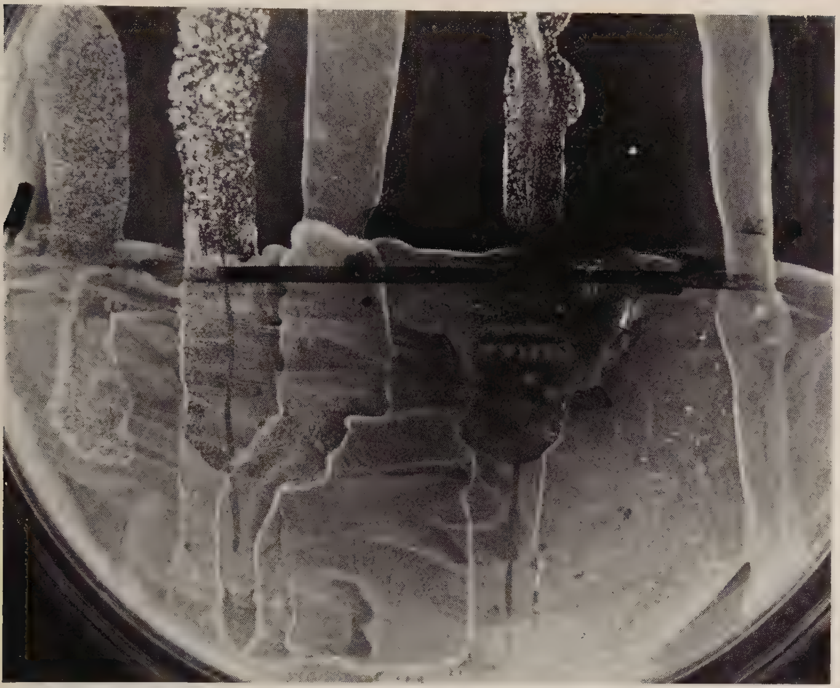

Fig. 6. A plate half of which was smeared with the cells of culture 22031 and allowed to grow before drops of cell suspensions from 5 different cultures were permitted to flow over the background growth. Three long streaks are visible due to the overflow, one from the second, one from the fourth and one from the fifth drop. × 1.3.

be due to travel over the agar of a mass of agglutinated viral cell debris leaving a trail of lysis behind it.

Multiple Budding.

An interesting phenomenon, encountered in some of the infected cultures within the first few hours of growth, is multiple budding. A healthy yeast cell produces a single bud which attains the size of the mother cell in about 90 minutes under favorable conditions. In cells infected with virus, or known to carry the virus, multiple budding is often observed (fig. 7). This is interpreted to mean that the virus interferes with some growth process but not with the initiation of a new bud, and when the growth of the first bud has been inhibited by the virus a second bud is produced before the first one is fully grown and that the succeeding buds all cease to

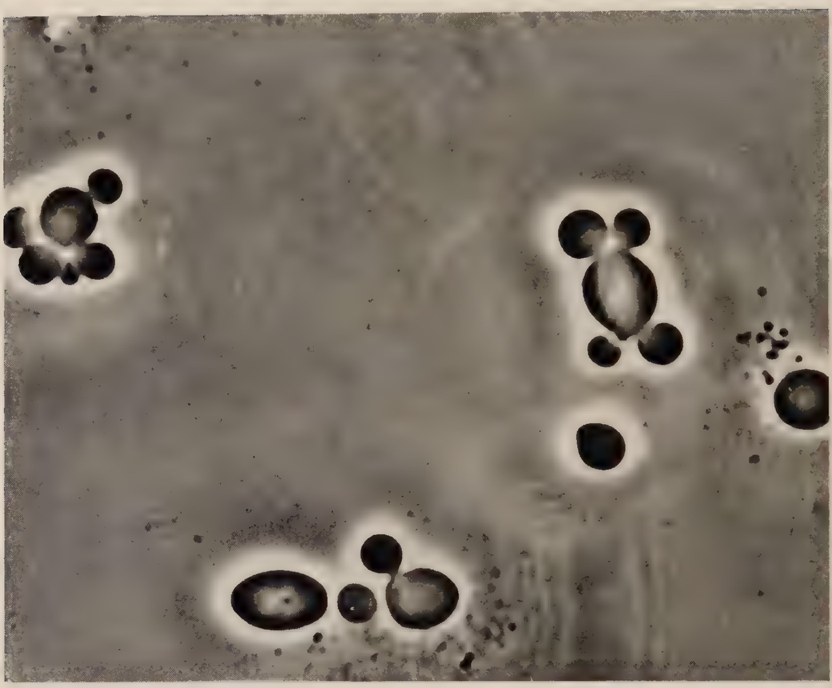

Fig. 7. Cells from a 24-hour broth culture of 22031 showing multiple buds probably due to viral infection. × 2600.

grow at the same stage of development. Multiple buds are only observed within the first 24 hours after transfer to broth. Some of the multiply budded aggregates have been observed to undergo lysis.

Lysis of Small Colonies.

When a culture infected with zymophage is plated on agar, the colonies vary considerably in size and many of the small colonies lyse, usually before the second day (fig. 8). When the small colonies are transferred from agar to broth within 12 hours, only a small fraction grow in the broth to produce turbidity. On some occasions not a single small colony from 60 or 100 transplants survived; on other occasions as many as 50 per cent have produced turbid growth. In the first test, 60 small colonies from one plate were transferred to nutrient agar after 24 hours, using a nichrome needle, and none of the subcultures grew. On the possibility that the nichrome wire might have exerted some toxic effect, a second attempt was made

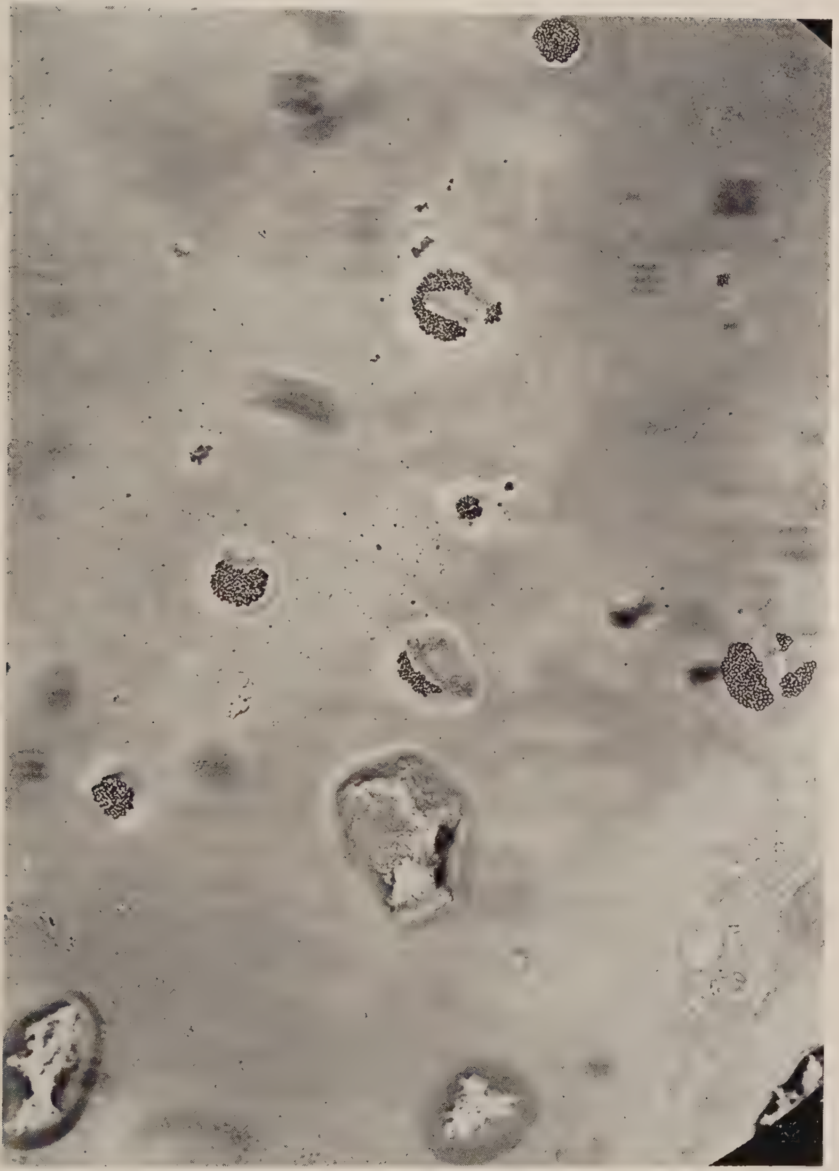

Fig. 8. Complete and partially lysed yeast colonies. This *status quo* may be reached before 48 hours and be maintained for a week or more without change. × 160.

using a platinum needle, but still no subcultures grew. A few days later, older colonies were transferred with the platinum needle and nearly all produced viable subcultures.

To avoid the use of metal needles, colonies were picked from another culture which had produced plaques, using a melting point tube drawn out to a capillary pipette. The capillary (full of cells) was broken off in a tube containing 2 ml of nutrient broth. After 5 days, 50 per cent of the small colonies, picked up on the first day, had produced subcultures, but the other tubes failed to grow after a much longer time. Over 90 per cent of the colonies picked on the second and third days produced subcultures. It was inferred that the small colonies picked on the first day failed to grow because they were infected. Older, larger colonies from the same plates could be transferred with considerable success. The difference between the viability of large and small colonies was confirmed by numerous separate experiments. In one experiment, for instance, only 20 per cent of the small colonies had produced turbidity after 16 days; 94 per cent of the large colonies produced viable subcultures (85 per cent of the large colonies had produced subcultures by the fifth day).

Transmission of the Infection.

The transmission of the infection from small to large colonies was demonstrated by mixing large and small colonies. Cells from a small colony were planted in the same broth tube with cells from a large colony. This procedure frequently prevented the cells from a large colony from producing turbidity. Fig. 9 shows the results of such an experiment. One hundred small colonies were transferred to 2 ml of broth from an agar plate which had been inoculated twelve hours previously. Eighty-eight small colonies failed to produce subcultures; twelve produced subcultures; eleven of these had produced subcultures by the third day. Fifty large colonies were transferred. Forty-nine (98 per cent) produced subcultures by the eighth day (90 per cent had produced subcultures by the third day). One hundred tubes were inoculated with one large and one small colony each. Seventy-eight of these produced turbid growth while 21 failed to produce turbidity. Therefore, the addition of cells from a small colony to a broth tube together with cells from a large colony prevented the cells of some of the large colonies from growing. This proved that the small colony, which is usually destined to

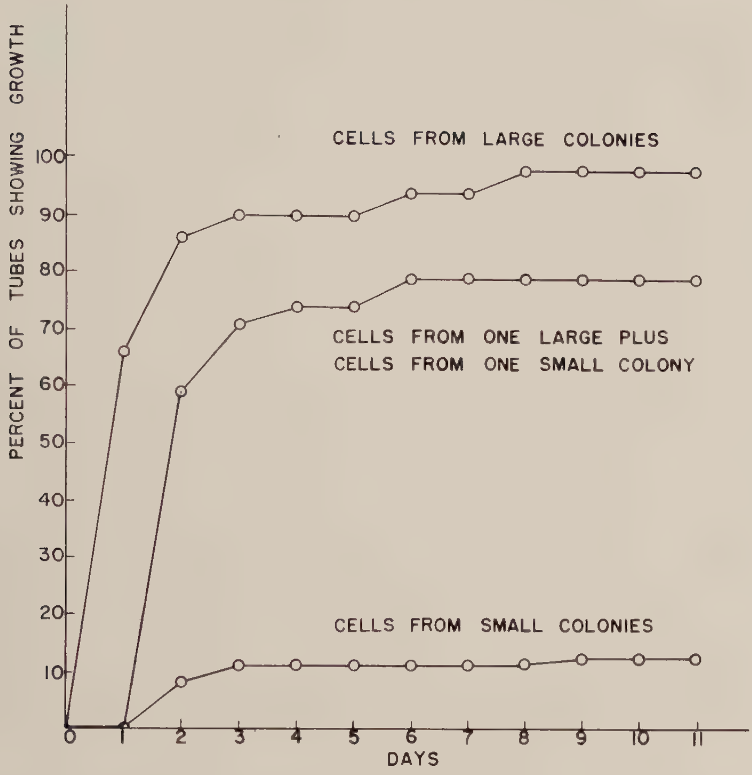

Fig. 9. A graph showing the percentages of broth tubes showing growth inoculated with (1) large, (2) small and (3) large plus small colonies, respectively.

undergo lysis, contains a virus which prevented all the cells of the large colony from growing in the broth tube.

Delayed Lysis.

In the later stages of growth on an agar plate a delayed lysis occurs in the uppermost layer of cells in the colonies. This phenomenon was first observed by Pisova in 1930 and confirmed by Lindegren and Hamilton in 1944. A similar lytic phenomenon occurs in the cells growing in thin films on an agar plate and has been especially conspicuous in the phage bearing cultures; in regions where a shallow growth of cells is present, due to the spreading of late growth from colony, the delayed lysis results in clearing of the shallow areas. The relation of delayed lysis to zymophage infection has not been clarified.

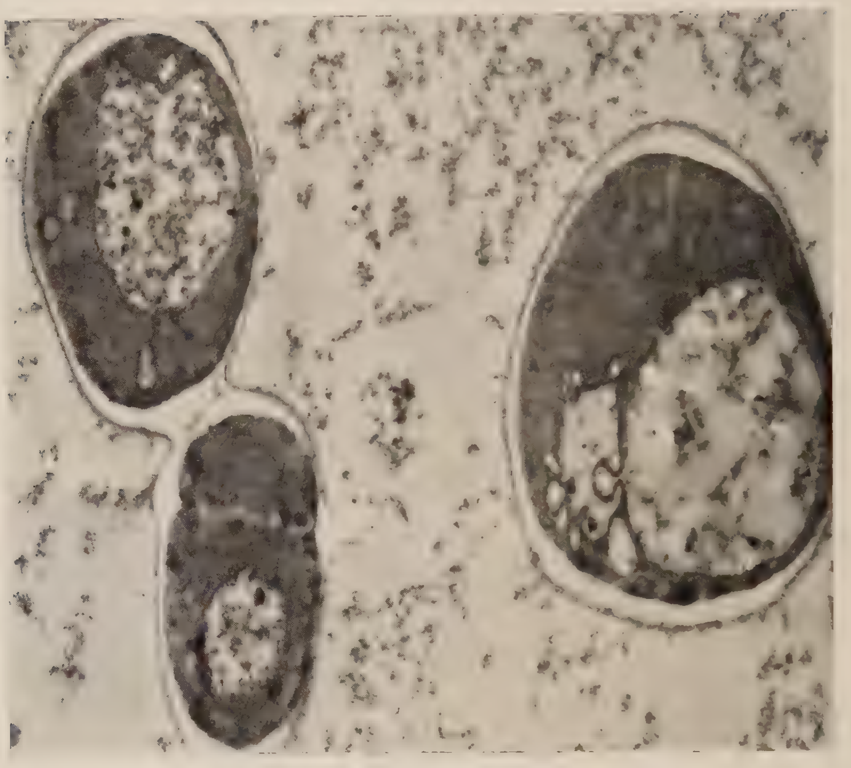

Fig. 10. An electron micrograph of an ultrathin section of a normal yeast cell. Normal yeast cells generally reveal a fine grained cytoplasm and do not contain granules of the type shown in Fig. 11. × 25700.

Electron Microscopy.

Dr. TADASHI HIRANO has kindly made a series of electron micrographs of preparations from virus-infected cultures. The zymophage particles (approximately 0.1 micron in diameter), present in the infected cultures, are not found in normal cultures. Electron micrographs of normal yeasts have been prepared by LINDEGREN and HIRANO (unpublished results). The conspicuous items are (1) the spindle, (2) the nuclear vacuole, (3) the chromosomes, (4) the nucleolus, (5) the fine-grained cytoplasm, (6) the plasma membrane, (7) the cell wall and (8) the mitochondria. The electron micrographs shown in Figs. 10 and 11 were prepared by fixation of cells in 2 per cent potassium permanganate fixative followed by post fixation treatment with 1 per cent uranyl acetate after which the cells were washed, dehydrated and embedded in methacrylate according to

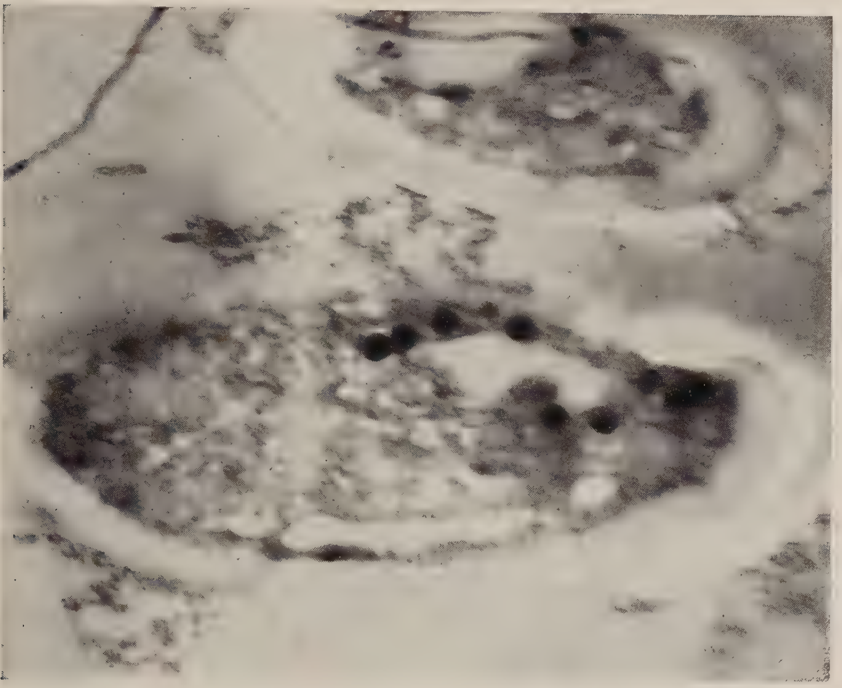

Fig. 11. An ultrathin section of a yeast cell from culture 22031 which produced the plaques shown in Fig. 1. This culture showed other symptoms of virus infection, such as multiple budding and disintegration of small colonies. The small dark granules (approximately 0.1 micron in diameter) and the coarse-textured cytoplasm are constant characteristics of virus-infected cultures. × 44400.

the method of MERCER (1959). The normal cells in Fig. 10 show a fine-grained cytoplasm. The spindle is visible in the original photograph but does not differ conspicuously from the cytoplasm and may not be perceptible in the reproduction. Uranyl acetate treatment which increases the visibility of virus particles appears to diminish the contrast between the cytoplasm and other cellular components. A turgid vacuole and cytoplasmic particles, possibly mitochondria, are visible in the normal cells. The infected cell in Fig. 11 is strikingly different. The cytoplasm is coarsely granular and seems to be coagulated; the vacuole is shrunken; there is no evidence of a spindle; and small, dense particles of the zymophage surround the vacuole. The cell in Fig. 11 arose from culture 22031 which produced the plaques shown in Fig. 1. It was obtained by the following procedure:

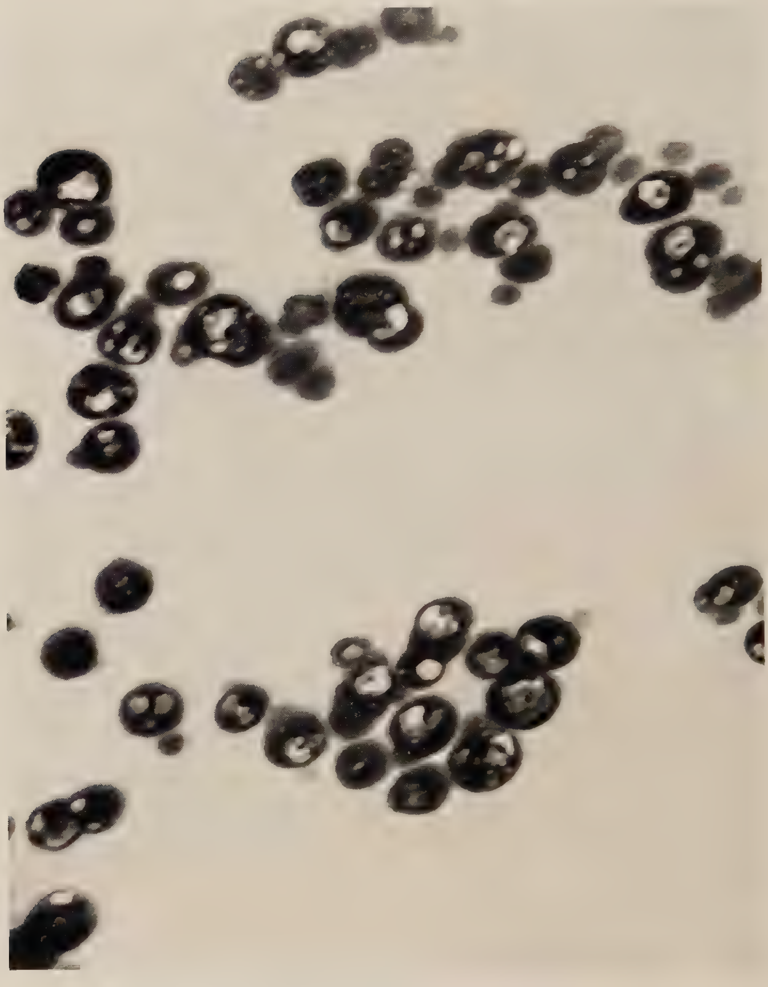

Fig. 12. An electron micrograph showing a field crowded with zymophage granules. × 43000.

A small colony (presumably infected with virus because it was smaller than the neighboring colonies) of culture 22031 was incubated 18 hours in broth and the suspension centrifuged at 15,000 rpm for 1 hour. The supernatant was filtered through a millipore filter and 1 ml of the filtrate was added to 2 ml of nutrient broth which was simultaneously inoculated with cells grown from a large colony of 22031 (The large colony was assumed not to be infected with virus). After 18 hours incubation the cells were col-

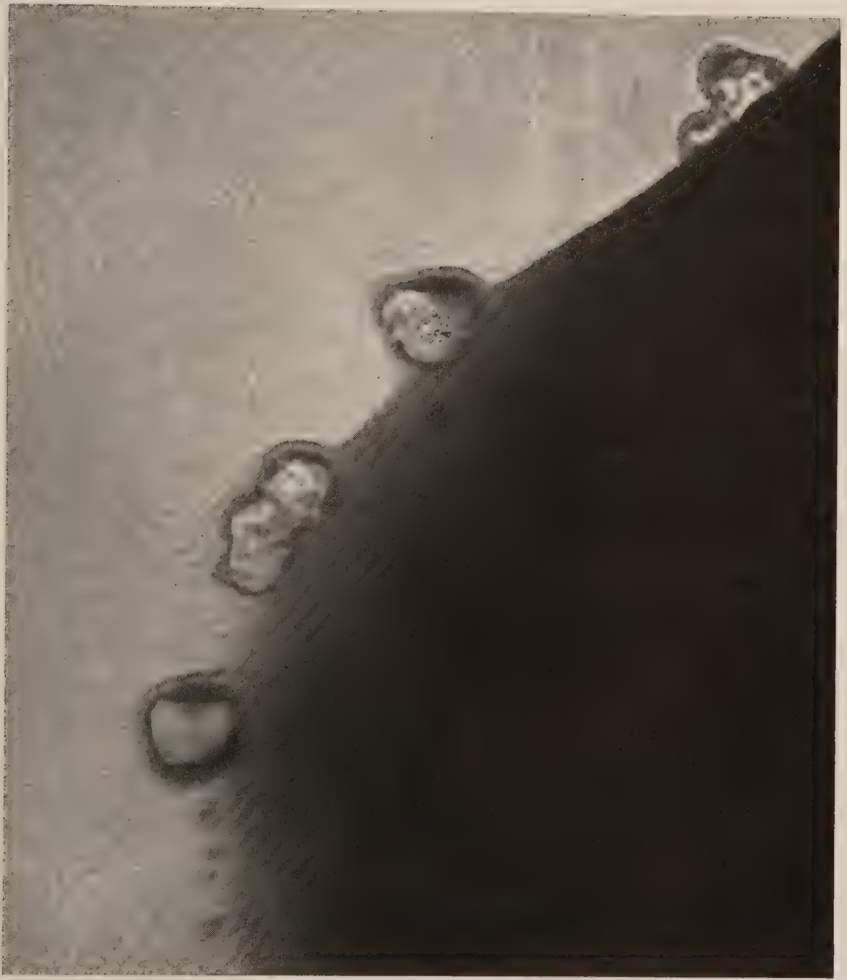

Fig. 13. Electromicrograph of the periphery of a whole unsectioned cell from culture 26848, showing attached zymophage. × 150000.

lected and washed and prepared for electron microscopy by Mercer's method.

The granules shown in Fig. 12 arose from culture 26926 and are approximately the same size as those shown in Fig. 11. Culture 26926 was inoculated into nutrient broth for four hours and the supernatant collected and placed directly on a collodion film. Fig. 13 shows the periphery of a whole yeast cell from culture 26848. The bodies on the periphery of the cell are comparable in size to those in

Figs. 11 and 12. In Fig. 11 they are inside the cell, in Fig. 12 they are free in the medium, and in Fig. 13 they are attached to the periphery of a yeast cell. The preparations shown in Fig. 12 and 13 were made by placing a small drop of suspension directly on the surface of a collodion film on a copper screen without either chemical treatment or sectioning.

Summary.

The capacity of the zymophage to transmit infection from yeast culture to yeast culture was demonstrated by mixing infected with uninfected cells. Various symptoms of zymophage infection are described. The zymophage was photographed by electron microscopy.

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ETUDE COMPAREE DE DEUX SOUCHES DE *CL. TETANI* ET DE LEURS FORMES L (COLONIE B), OBTENUES PAR L'ACTION DE LA GLYCINE

par

C. GONZÁLEZ

(Reçu le 24 Mai 1960).

Dans un travail précédent (GONZÁLEZ et RUBIO, 1959) nous avons décrit l'obtention des formes L de *Clostridium tetani* induites par la glycine, semblables à celles obtenues par DIENES en 1950 de la même bactérie induites par la pénicilline. En plus des éléments morphologiques qui caractérisent chaque état du cycle "L" nous avons obtenu une forme nouvelle, qui, à notre connaissance n'a été décrit par aucun des auteurs qui ont étudié le cycle "L" des bactéries Gram positifs ou Gram négatifs.

Le nouveau type morphologique (c'est-à-dire, les éléments sphériques) s'est toujours conservé même après une centaine de repiquages dans les milieux usuels, exempt de glycine. Les colonies constituées par ces éléments sphériques nous les avons appelées forme L (colonie B) de *Cl. tetani*. Les corpuscules sphériques de la colonie B sont pathogènes pour les souris et les cobayes, reproduisant le syndrome classique du tétanos par inoculation de la toxine ou bien par inoculation des éléments mentionnés préalablement lavés. Nous avons isolé les mêmes corpuscules sphériques de souris mortes, qui avaient été préalablement inoculées avec eux.

Nous faisons dans ce travail une étude comparée morphologique et biochimique de deux souches normales de *Cl. tetani* et de leurs formes L respectives (colonie B), et nous titrons les toxines produites par l'une et l'autre forme.

MATERIALS ET METHODES.

Milieux de culture.

Nous avons employé les milieux complexes d'usage courant pour leur identification (LEBERT et TARDIEUX, 1952). Les résultats indiqués ci-dessous ont été obtenus sur milieux de culture identiques sans glycine et dans les mêmes conditions d'incubation, car nous avons observé que la colonie B une fois obtenue se maintient durant un certain temps avec ses caractères spéciaux dans le milieu sans glycine.

Souches utilisées.

Nous avons utilisé les mêmes souches que dans notre travail précédent (GONZÁLEZ et RUBIO, 1959): *Cl. tetani* P. envoyée par le Professeur PREVOT de l'Institut Pasteur de Paris, et *Cl. tetani* I, due à la bienveillance du Dr. OTEGUI de l'Institut Ibys de Madrid et leurs formes L (colonie B) respectives.

RÉSULTATS.

Morphologie.

L'aspect macroscopique de la colonie B cultivée en profondeur en tubes de milieu gélosé est semblable à celui de la forme normale: lenticulaire, composée, cotonneuse, de 1,5-2,5 mm de diamètre. Les corpuscules sphériques qui la forment, mesurent 2,5-4μ de diamètre, sont groupés en grappes, immobiles, sans spore, fortement G positifs.

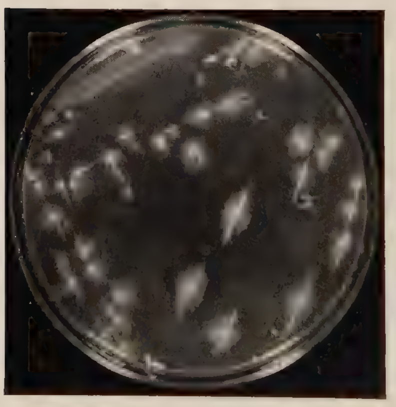

Fig. 1. Culture de *Cl. tetani*, forme normale.

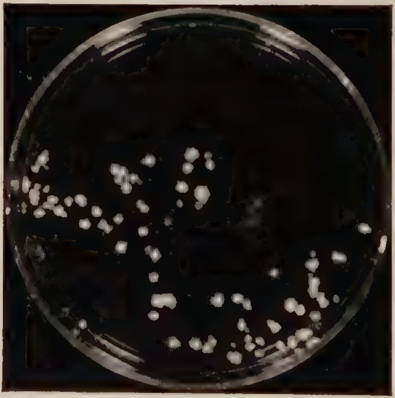

Fig. 2. Culture de *Cl. tetani*, forme L (colonie B).

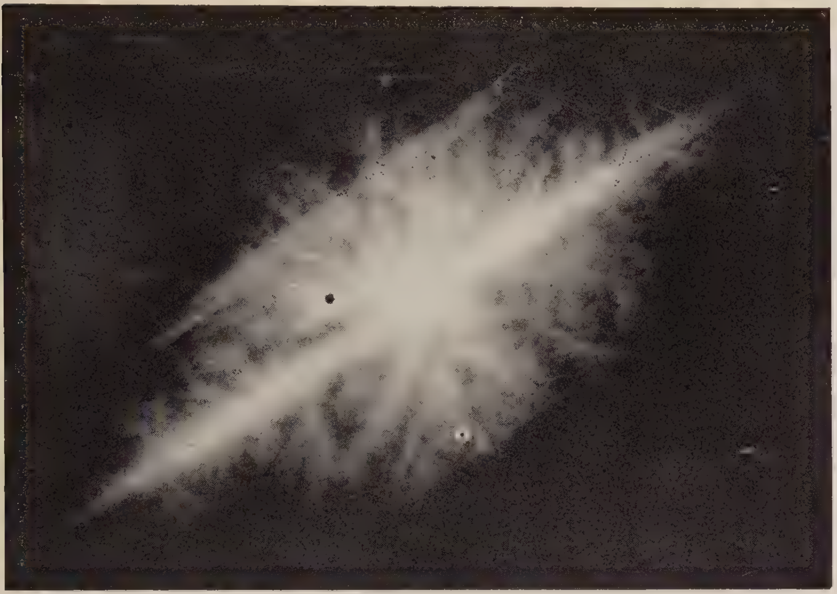

Fig. 3. Colonie normale de *Cl. tetani*.

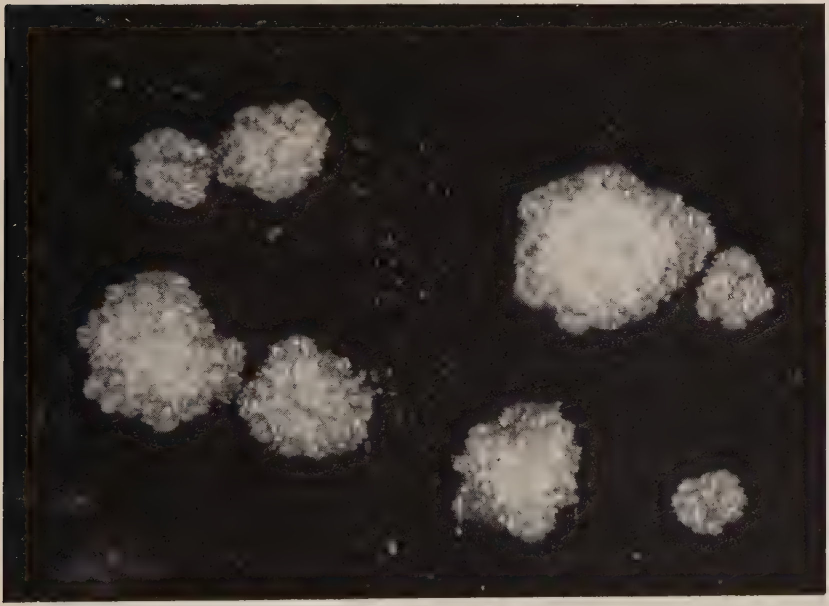

Fig. 4. Colonie B de *Cl. tetani*.

Les colonies en surface en boîtes de Petri incubées en pots de Brewer, sont quelque peu différentes des colonies normales respectives. Elles sont comme celles-ci irrégulières, mais plus compactes, plus élevées sur le milieu de culture, avec la surface totalement mamelonnée, brillantes et plus petites, 2-4 mm de diamètre (Fig. 1, 2, 3 et 4).

Physiologie.

Les éléments de la colonie B de même que ceux de la forme normale sont strictement anaérobies et réducteurs; un peu moins résistants à la chaleur, ils meurent après 5 minutes à 80° C., mais ils tolèrent une minute à cette température. Ils produisent du gaz en milieu solide et liquide et les cultures sont fétides.

Les cultures en milieu VF, VF avec glucose et en eau peptonnée croissent au fond du tube sous forme de grumeaux fins et compacts, tandis que le liquide surnageant reste tout à fait transparent se distinguant bien du trouble homogène de la forme normale. L'aspect de la culture en milieu liquide ressemble à celui de quelques Corynebactéria anaérobies.

Les deux formes ont le même comportement face à la gélatine, les protéines coagulées, le blanc d'oeuf coagulé, le lait et les glucides.

Biochimie.

Nous avons observé un comportement analogue pour les deux formes en ce qui concerne les produits de fermentation du milieu VF avec 10°/$_{oo}$ de glucose. Nous déterminons la production d'acides acétique, propionique et butyrique; des amines volatiles; d'ethanol et des traces de métacrésol. Ils produisent du NH_3 et du SH_2 en abondance. Nous n'avons observé de formation d'acétyl-méthyl-carbinol dans aucune des deux formes. Ils ne réduisent pas les nitrates en nitrites.

La courbe de croissance des formes L présente une période de latence plus longue que celle correspondant aux souches normales; généralement on ne perçoit aucune croissance des formes L avant 48 heures d'incubation.

Antibiogramme.

Comportement analogue pour les deux formes; halo d'inhibition de 24, 20, 18, 15 et 8 mm face à la terramycine (30 mcg), auréomycine (30 mcg), erythromycine (15 mcg), chloromycetine (30 mcg) et pénicilline (10 units).

Titrage de la Toxine.

Elle a été obtenue sur milieu VF avec 10°/₀₀ de glucose incubé à 37° C. en flacons.

Le titre de l'hémolysine est le même pour la forme L et pour sa forme normale respective: 1/1.000 pour la souche *Cl. tetani* I, et 1/100 pour la souche *Cl. tetani* P. Le titrage a été réalisé après 48 heures d'incubation, vu qu'il faut ce temps pour obtenir une culture des formes L dans la phase logarithmique. Si l'on fait les titrages avec des toxines de 24 heures de souches normales et avec des toxines de 48 heures de leurs formes L, le titre de l'hémolysine de ces dernières est un peu plus faible que celui des premières.

La détermination de la dose DM/50 selon Reed et Muench (1938) sur des souris de 18-20 g par inoculation souscutanée d'un ml de la dilution de toxine donne des valeurs de 31.600 DM/50 et 160.000 DM/50 par ml pour la toxine synthétisée par la forme normale de *Cl. tetani* I, et pour celle de sa forme L. La toxine produite par *Cl. tetani* P. et celle produite par sa forme L, ont le même titre: 53.000 DM/50 par ml.

D'après ces résultats, le comportement physiologique et biochimique des formes L (colonie B) de *Cl. tetani* semble similaire à celui de sa forme normale. En considérant que nous nous sommes bornés dans nos expériences à des déterminations qualitatives et non pas quantitatives qui nous permettraient de fixer cette conclusion comme définitive, les faits, malgré cela, semblent appuyer les recherches de Medill et O'Kane (1954), et celles de Minck, Kirn et Galleron (1957) qui ne trouvèrent pas dans les microorganismes étudiés par eux, des changements métaboliques fondamentaux durant la transformation L.

Discussion.

Dans notre travail précédent dont le résumé a été présenté au Symposium sur la Biologie des "Pleuropneumonia-like organisms", New York Academy of Sciences (Rubio et González, 1959) nous avions annoncé l'obtention des formes L (colonie B) de *Cl. tetani* capables de produire une toxine au moins du même titre que celui de leur forme normale respective. Dans la discussion de ce travail, Freund (1960) confirme l'obtention dans ses laboratoires, par Scheibel et Assandri (1959), dans des expériences analogues aux nôtres avec quatre souches de *Cl. tetani*, des formes L induites par la pénicilline, trois d'entre elles paraissant aussi pathogènes que les souches normales respectives et même un peu plus. Dans le travail de Scheibel et Alessandri (1959) on observe que le titre de la toxine des formes L est 10 fois plus élevé que celui des formes normales.

Dans nos expériences avec une des formes L d'une des souches employées (*Cl. tetani* I) la toxine possède un titre à peu près 5 fois plus élevé que celui de sa forme normale. Cette différence en faveur de la forme L se conserva constante durant toute la phase de synthèse de la protéine toxique, ainsi que le prouvent les titrages partiels faits après 48, 120, 144, et 168 heures d'incubation, ce dernier temps étant celui auquel le titre arrive au maximum, tant dans la forme normale que dans la forme L respective. Par contre la souche *Cl. tetani* P. synthétise une toxine dont le titre ne présente pas de différences avec celui de sa forme L respective.

Cette légère différence dans le titre de la toxine de l'une des formes L avec celui de la souche normale, ne mérite pas, à notre avis, d'être considérée comme une modification du métabolisme durant la phase de transformation L, si nous considérons surtout que la forme L de l'autre souche étudiée ne présente aucune différence avec sa forme normale. Nous croyons pouvoir expliquer cette légère anomalie par le fait suivant: durant toute la durée de notre expérience la forme L de la souche *Cl. tetani* I était dans un état de transformation intégrale, au moins à l'observation microscopique; on pouvait observer que les cultures étaient entièrement formées par des corpuscules sphériques qui se conservèrent jusqu'à la fin de l'expérience; par contre la souche *Cl. tetani* P. avait un peu évolué; on pouvait observer que les corpuscules sphériques alternaient avec des formes filamenteuses qui se trouvaient à la fin de l'expérience dans une proportion supérieure de 50% par rapport aux formes sphériques.

Le mécanisme intime de l'action de la glycine sur les bactéries, différent de celui de la pénicilline, n'est pas encore tout à fait élucidé (Welsch et Osterrieth, 1958), mais les auteurs sont d'accord pour admettre comme conséquence une altération ou une perte du mécanisme responsable de la synthèse de la paroi cellulaire. Ce fait et celui de ne pas avoir observé de changements métaboliques marqués dans les formes L, par nous étudiées, par rapport à leurs formes normales nous conduisent à penser que le titre un peu plus élevé de la toxine synthétisée par la forme L en état de transformation totale, pourrait être dû simplement, à un phénomène de diffusion dû à une augmentation de la perméabilité de la cellule pour la toxine synthétisée en elle.

Nous remarquons que contrairement à ce qui s'admet, nous avons trouvé dans nos expériences que la résistance contre la pénicilline de deux formes L (colonie B) étudiées, est analogue à celle des formes normales. Ceci peut être expliqué si nous tenons compte que la forme L (colonie B) de *Cl. tetani* a été obtenue par la glycine et non par la pénicilline.

Les éléments qui constituent cette colonie nous les considérons comme nous l'avons noté dans la discussion de notre travail précédent (GONZALEZ et RUBIO, 1959), comme un nouveau type morphologique du cycle L de *Cl. tetani* que nous appelons colonie B. Ce terme nous préférons l'employer tant que nos travaux en cours n'auront pas précisé les rapports entre ce nouveau type et la forme L classique de *Cl. tetani*.

Résumé.

Les propriétés de deux souches de *Cl. tetani* et de leurs formes L obtenues par l'action de la glycine sont comparées.

Le titre de la toxine synthétisée est déterminé et il paraît que celui de l'une des formes L est un peu plus élevé que celui de sa forme normale, tandis que l'autre souche ne présente pas de différences dans le titre de ces deux formes.

Ni des différences physiologiques ni biochimiques marquées n'ont été observées entre les formes normales et leurs formes L respectives, ni une sensibilité différente vis à vis des antibiotiques.

Summary.

A comparative study of two strains of *Cl. tetani* and its L forms obtained by glycine action has been carried out.

The titer of the toxin synthesized by the L forms of one of the strains was slightly higher than by its normal form but there was no difference between the titer of the toxin synthesized by the L and the normal form of the other strain. We conclude from our results that there are no fundamental biochemical differences between the normal and L forms.

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(The Marine Laboratory, University of Miami, Miami, Florida, U.S.A.).

A NEW SPECIES OF *SACCHAROMYCES* ISOLATED FROM A SUBTROPICAL ESTUARY [1] [2]

by

JACK W. FELL [3]

(Received August 20, 1960).

Our recent investigations (Fell *et al.*, 1960) of the microflora of Biscayne Bay, Florida, have demonstrated the presence of various species of yeasts in this estuarine locality. Included in these collections was an organism identified as a previously undescribed species of *Saccharomyces*. This organism was isolated in April, 1958, from sediment taken at a water depth of 6 ft., approximately 300 yds. from shore. The salinity range at this site is 18—$31^0/_{00}$.

Taxonomic examination of the isolate followed the procedures of Lodder and Kreger-van Rij (1952) and Wickerham (1951). The liquid carbon assimilation test of Wickerham (1951) was employed. Vitamin requirements were established by the methodology of van Uden and Farinha (1958).

Description of *Saccharomyces aestuarii* nov. spec.

The vegetative cells are haploid. Conjugation occurs between the mother and daughter cell. One to four round ascospores are formed per ascus (Fig. 1). Sporulation is abundant after 2—3 days on malt extract-yeast extract medium or malt extract medium (Wickerham, 1951). The colonies are smooth and dull white producing a diffusible red non-carotenoid pigment.

After three days growth in liquid malt extract the cells are round to oval (3—5) × (4—7) μ. A primitive pseudomycelium is formed on corn meal agar.

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[1] Contribution No. 291 from The Marine Laboratory, University of Miami.

[2] This study was developed under Grant G-6367 from the National Science Foundation.

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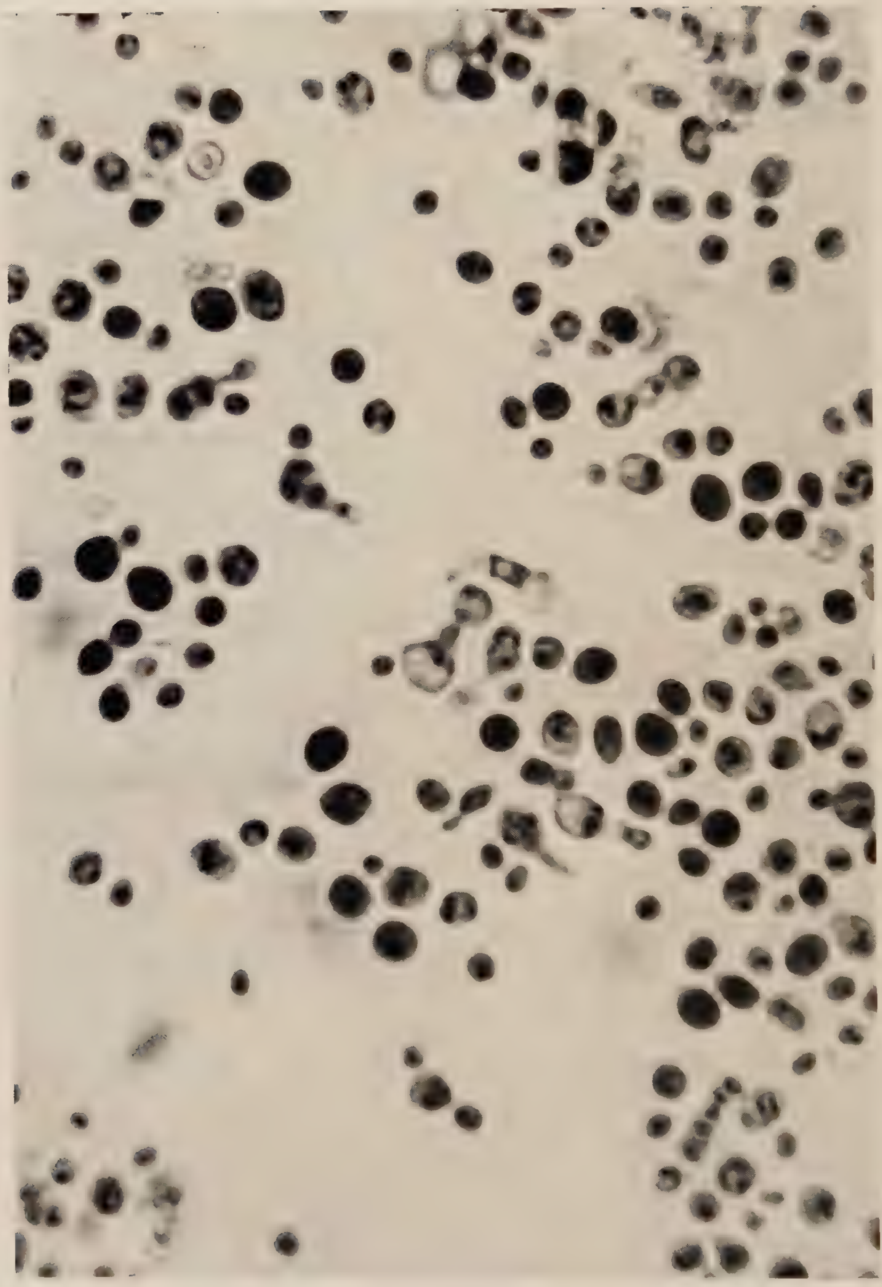

Fig. 1. Ascospore formation in *S. aestuarii* following conjugation between mother and daughter cells (1000 ×).

Glucose, sucrose and raffinose (1/3) are fermented, while galactose, lactose, and maltose do not show fermentation.

Assimilation is positive for glucose, galactose, *L*-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, raffinose, ethyl alcohol, glycerol, *D*-mannitol, *D*-sorbitol, salicin, pyruvic acid, lactic acid, and succinic acid.

Assimilation is weak to variable in melezitose, and xylose.

Assimilation is absent in melibiose, inulin, soluble starch, *L*-arabinose, *D*-arabinose, *D*-ribose, *L*-rhamnose, *D*-glucosamine hydrochloride, i-erythritol, adonitol, dulcitol, alpha-methyl-*D*-glucoside, potassium *D*-gluconate, calcium 2-keto-*D*-gluconate, potassium 5-keto-*D*-gluconate, citric acid, ethyl acetoacetate, and i-inositol.

Tryptophan is not required for growth.

Assimilation of KNO_3 is negative.

There is no growth at 37°C.

Growth is absent in a vitamin-free medium; a growth requirement for niacin is indicated.

This species seems to be closely related to *S. lactis*; it differs from this species by failure to ferment lactose and galactose.

The type strain of this species, designated MY-111, is maintained in the culture collections of The Marine Laboratory, University of Miami, Miami, Florida, The Northern Utilization Research and Development Division, Agricultural Research Service, Peoria, Illinois, and in the Centraalbureau voor Schimmelcultures, Delft, Holland.

Latin diagnosis:

Saccharomyces aestuarii nov. spec.

In musto maltato cellulae rotundae aut ovoideae, (3–5) × (4–7) μ. Pseudomycelium primitivum formatur. Cellulae vegetativae haploideae. Conjugatio heterogamica inter cellulas maternas et earum gemmas. Ascosporae rotundae; 1–4 in asco. Formantur coloniae glabrae, albidae, non-nitidae. Pigmentum non-carotinoideum in medio diffundit. Fermentatio glucosi, sacchari et raffinosi pro tertia parte; galactosum, lactosum et maltosum non fermentantur. Assimilat glucosum, galactosum, *L*-sorbosum, maltosum, saccharum, cellobiosum, trehalosum, lactosum, raffinosum, alcohol aethylicum, glycerinum, *D*-mannitolum, *D*-sorbitolum, salicinum, acidum pyruvicum, acidum lacticum et acidum succinicum. Assimilatio melibiosi et *D*-xylosi variat. Non assimilat melibiosum, inulinum, amylum, *L*-arabinosum, *D*-arabinosum, *D*-ribosum, *L*-rhamnosum, *D*-glucosaminum hydrochloricum, *i*-erythritolum, adonitolum, dulcitolum, alpha-methyl *D*-glucosidum, potassium *D*-gluconatum, calcium 2-keto-*D*-gluconatum, potassium 5-keto-*D*-gluconatum, acidum citricum, acetoacetatum aethylicum et *i*-inositolum. Nitras kalicus non assimilatur. Non crescit 37°C.

Acknowledgements.

The author wishes to thank Dr. L. J. WICKERHAM, Northern Utilization Research and Development Division, Peoria, Illinois, for his extensive examination of this species and Mrs. KREGER-VAN RIJ, Centraalbureau voor Schimmelcultures, Delft, Holland, for her confirmation of our studies. The advise of Dr. N. VAN UDEN, Department of Microbiology, University of Lisbon, Portugal, Dr. S. P. MEYERS, The Marine Laboratory, and Dr. F. J. ROTH, Jr., Department of Microbiology, University of Miami is appreciated.

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A NEW *SALMONELLA* TYPE (*S. BENGUELLA*)

by

P. A. M. GUINÉE and E. H. KAMPELMACHER

(Received August 1, 1960).

In June 1959 a culture was isolated from fish-meal of Portuguese Angola origin by Dr. H. Esseveld (Bact. Laboratory, Gemeente Ziekenhuis, Rotterdam).

After examination of single colonies it was shown, that this culture was a mixture of *S.meleagridis* and the new type *S.benguella*. The new type showed the morphological and biochemical characteristics of the genus *Salmonella*, *i.c.* Gram-negative, motile rods, no growth in Møller's KCN-medium, rapid fermentation of glucose (with gas), mannitol, dulcitol, inositol, arabinose, rhamnose, trehalose and xylose, utilisation of citrate, mucate, *d*- and *i*-tartrate and production of H_2S. Gelatin was not liquefied, indol was not formed, urea was not hydrolysed and salicin, lactose, sucrose and adonitol were not fermented. The Voges-Proskauer test was negative and the methylred test positive.

The organism was agglutinated to titer (1 : 1280) by *S.allendale* O serum; in absorption tests all agglutinins were removed from the serum. An O-serum prepared with the new type, agglutinated to the same titer the homologous strain and *S.allendale*. The O agglutinins were all removed by *S.allendale*. The culture therefore belonged to O-group S and possessed the somatic antigen 40 of the genus *Salmonella*.

The H-antigens were diphasic. Phase 1 was agglutinated to titer (1 : 10.000) by *S.paratyphi* B (b) and phase 2 was agglutinated by *S. kentucky* (i : z_6).

In absorption tests all agglutinins of these two sera were removed. It can therefore be concluded, that *S.benguella* is a hitherto undescribed *Salmonella* type with the antigenic formula 40 : b : z_6.

We are indebted to Dr. Kauffmann for confirmation of our findings.

Summary.

A new *Salmonella* type *S.benguella*, with the antigenic formula 40 : b : z_6, is described. The culture was isolated together with *S.meleagridis* from fish-meal of Portuguese Angola origin.

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CONTINUOUS HYDROXYLATION OF PROGESTERONE BY *ASPERGILLUS OCHRACEUS* [1] [2]

by

RICHARD I. MATELES and GEORGE J. FULD [3]

(Received October 4, 1960).

INTRODUCTION.

In recent years the use of continuous fermentation in both industry and research has been the subject of reviews by workers in several countries (7, 11, 12, 16, 19, 22). In his review, MAXON (19) classifies fermentations into three categories: Type I, in which the rate of product formation is substantially identical to the rate of growth of the micro-organism, exemplified by the production of food yeast; Type II, in which the rate of product formation is related to the growth rate only indirectly, exemplified by ethanol and organic acid production; and Type III, in which the rate of product formation appears to have no relationship to the growth rate of the micro-organism, exemplified by the antibiotic, steroid, and vitamin fermentations. Type III fermentations are of the greatest importance industrially, and yet have been investigated the least with continuous culture techniques.

The subject of the investigation described herein is a Type III fermentation involving a mycelium-producing organism: the hydroxylation of progesterone by *Aspergillus ochraceus* NRRL 405 to give 11α-hydroxyprogesterone and then 6β, 11α-dihydroxyprogesterone (9, 13). In addition to being Type III, the transformation of the steroid produces two products, one of which, the 11α-hydroxyprogesterone, was considered as the desired one in the present studies. The investigation had three purposes: (i) To develop a fermentation system capable of growing continuously a mycelium-producing micro-organism; (ii) to use this system to hydroxylate progesterone continuously; and (iii) to determine whether a two-stage system would be necessary in order to obtain a maximum yield of 11α-hydroxyprogesterone with respect to the other reactants.

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It should be emphasized that this study was not undertaken to produce greater yields of steroids more rapidly – rather, the choice of the steroid fermentation was secondary to the study of the kinetics of a Type III fermentation. The steroid transformation was chosen since it could be studied relatively easily, with a minimum of by-product formation. It is hoped that through studies such as reported here, additional data as to the kinetics of complex fermentations can be obtained, and that eventually the understanding of these kinetics will enable the improvement of industrial fermentation productivity.

The theoretical aspects of continuous culture are adequately described in the literature (7, 11, 12, 20, 21). The nomenclature used in this paper is summarized below:

μ = specific growth rate, hours^{-1}.
μ_m = maximum growth rate, hours^{-1}.
x = organism concentration, grams/liter.
t = time, hours.
s = concentration of limiting nutrient, grams/liter.
s_r = concentration of limiting nutrient in influent stream, grams/liter.
D = dilution rate, hours^{-1}.
Y = yield value, grams mycelium/gram substrate consumed.
T = contact time in fermentor, hours.

Subscripts 1 and 2 refer to the first and second-stage fermentors, respectively.

Materials and Methods.

Equipment.

Fig. 1 is a diagrammatic representation of the fermentor system arranged for continuous growth and hydroxylation. There are three basic units: two fermentors, of which the second can be removed if one-stage operation is desired; a nutrient reservoir and pump for

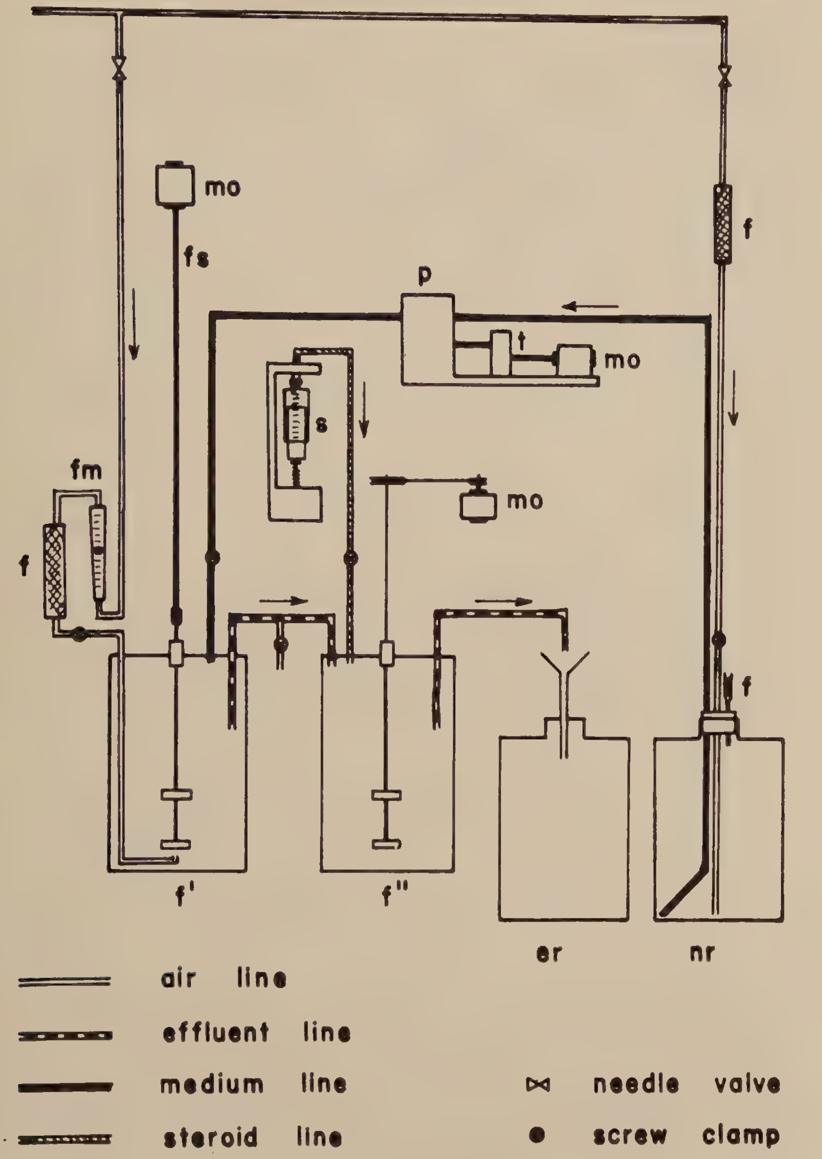

Fig. 1. Two-stage continuous fermentation system with steroid addition to second stage (diagrammatic). *er* = effluent reservoir. *f* = air filter. *f'* = first-stage fermentor. *f''* = second-stage fermentor. *fm* = air flowmeter. *fs* = flexible shaft. *mo* = motor. *nr* = nutrient reservoir. *p* = Sigmamotor pump. *s* = kymograph-driven syringe. *t* = transmission unit.

continuously adding nutrient medium to the first-stage fermentor; and a means for continuously adding progesterone solution to either the second-stage fermentor (shown) or the first-stage fermentor.

The fermentors, of five-liter size, were similar to those described by Bartholemew *et al.* (1) and were obtained from Artisan Metal Products, Inc., Waltham, Massachusetts. The first and second stages, if two stages were used, were connected with gum rubber tubing and a "*T*" connection for taking samples of the first-stage fermentor's effluent. The two fermentors were almost identical, the only differences being the type of seal used for the impeller shaft bearing and the fact that the sparger of the second-stage fermentor was removed. The fermentors were constructed so as to minimize surfaces to which mycelium might adhere. No thermometer wells were used, and sufficient space was left between the baffles and the fermentor walls so that mycelium could pass between without being trapped. The tubes through the tops of the fermentors were closed with gum rubber serum-bottle caps which permitted the addition of nutrient medium, progesterone solution, or other liquids through hypodermic needles. All metal parts of the fermentors were made of 316 stainless steel. The wall and bottom of each fermentor was a Pyrex cylindrical jar of five liters capacity. However, the usual volume of medium, contained in the first-stage fermentor, was 3.0 liters and in the second-stage fermentor 1.4—1.7 liters.

Air was supplied to the first-stage fermentor, which was stirred at 540 r.p.m., through a sparger at the rate of 3.0 liters/minute. The air passed through a flowmeter and then through several inches of packed glass wool which sterilized it. Experimentally, it was found that it was not necessary to humidify the air, since there was a negligible moisture loss. The second-stage fermentor was aerated by the violent splashing due to the low liquid level and the high impeller speed of 980 r.p.m.

The first-stage fermentor was held at 30 ± 0.5°C. by a constant temperature bath in which it was placed. The second-stage fermentor was placed in a bath half-filled with water, but with no temperature control. The temperature of the second stage maintained itself at 30 ± 1°C. because a balance was reached between the heat input due to stirring and the heat dissipated through the walls into the water bath. The temperatures were determined on the effluent stream of each fermentor.

The medium addition system consisted of a nutrient medium reservoir and a Sigmamotor pump. The reservoir was a 20-liter Pyrex carboy sealed with a rubber stopper pierced by three Pyrex tubes. Through one tube nutrient medium was pumped into the first-stage fermentor. Through another tube, sterile air was bubbled into the carboy to keep the antifoam and other suspended matter evenly dispersed. The third tube was packed with glass wool to provide a sterile passage for air to enter the carboy as it cooled after sterilization and to leave it as air was bubbled in. The medium tube was connected by gum rubber tubing to a hypodermic needle which entered the first-stage fermentor through a serum-bottle cap. The tubing passed through the Sigmamotor pump which could be adjusted to give flow rates from 40—5000 ml/hr using 1/8″ I.D. tubing.

The steroid addition system consisted of a 20 ml syringe, which held the progesterone solution, connected by polyethylene tubing to a hypodermic needle leading into the first- or second-stage fermentor. Neither Tygon nor gum rubber tubing could be used, as the progesterone solvent, N,N-dimethylformamide, softened or dissolved them. The plunger of the syringe was driven by an electric kymograph and attachment (Phipps and Bird., Inc., Richmond, Virginia), and the delivery rate used was 0.71 ml/hr. To prevent the solution from leaking between the plunger and the walls of the syringe and depositing progesterone crystals which caused jamming, the plunger was heavily coated with vaseline, which was sufficient to prevent jamming for about one day, at which time the dirty syringe was replaced with a clean one. The steroid addition system was not heat sterilized before use, but the solvent apparently killed any of the few micro-organisms present.

Progesterone addition.

The progesterone was added to the fermentor dissolved in N,N-dimethylformamide in a concentration sufficient to give a concentration in the fermentor of 250 mg/l. For instance, since the rate of addition of progesterone solution was 0.71 ml/hr, if the nutrient medium flow rate were 180 ml/hr, the concentration of progesterone in the solution would be (250)(.180)(.71) equal to 63 mg progesterone/ml solution. Carbon-14 labelled progesterone was incorporated into the unlabelled progesterone in the amount of 1—2 microcuries/gram. The progesterone used for the radioisotope dilution analysis was 4-C^{14} progesterone, with a specific activity of 10 μc/mg. This was for purposes of analysis as explained in a following section.

Steroid analysis.

Although previous workers have used chloroform (9, 13) or methylene chloride (24) to extract the steroids from the fermentation broth, ethyl acetate has been shown to be a better solvent for steroids containing several ketone or hydroxyl functions (3, 14). With ethyl acetate, 95—98% of the added steroid was recovered, and no trouble was experienced with emulsion formation.

The procedure used was as follows: Ten ml of broth and mycelium were extracted with four five-ml portions of ethyl acetate. The extracts were pooled and taken almost to dryness under a stream of nitrogen. The residue was taken up in one ml of ethyl ether, and an aliquot was streaked for chromatography on a 1″ strip of Whatman #1 filter paper impregnated with 1,2-propanediol (26,29). The strip was developed with toluene saturated with 1,2-propanediol, and the steroid spots located by using an ultraviolet scanner (8). The strip was dried for 15 minutes at 105°C. to remove most of the 1,2-propanediol, and the spots were cut out and eluted with 5 ml of methanol by shaking on a reciprocating shaker for two hours. A four ml aliquot of the methanol solution was plated on a planchet and dried for radioactivity measurements. The counts were made using a Tracerlab TGC-14, luminized Mylar, thin window, gas-flow Geiger tube and standard sample changing, scaling, and printout equipment. Each planchet was counted for a total of 2048 counts. Since the amount of material on the planchet was found to be less than 0.2 mg/cm^2, the counts were assumed to have been made at infinite thinness and no self-absorption correction was applied (4). The amount of progesterone, 11α-hydroxyprogesterone, and 6β, 11α-dihydroxyprogesterone in the sample was found by multiplying the amount of steroid in the broth (generally 250 mg/l) by the relative activity found in each spot on the strip. For instance, if the steroid concentration in the broth were 250 mg/l, and the activity contained in the spots was: progesterone, 21 c.p.m.; 11α-hydroxyprogesterone, 46 c.p.m.; and 6β, 11α-dihydroxyprogesterone, 7 c.p.m., all measured above the background of about 10 c.p.m., then the amount of 11α-hydroxyprogesterone in the broth would be (46/74) (250) equal to 155 mg/l. However, the quantities of steroids were reported on the basis of mg steroid/g mycelium, so the 155 mg/l would be divided by the mycelial concentration.

This method of steroid analysis, based upon the use of radioactive progesterone, was found superior to the method based upon spectrophotometric determination of the optical density at 240 mμ of the methanolic eluate (26), because of the fact that there were large amounts of ultraviolet absorbing substances in the paper which could not be completely removed by washing with methanol or toluene, as well as extraneous ultraviolet absorbing materials in the fermentation broth. Because of the low ratio of labelled to unlabelled progesterone used, the sensitivity of this method was not as great as that of the ultraviolet method, but the freedom from interfering substances and from the need to run blanks made the method more convenient and reliable. It should be realized also that since the absolute error in the determined amount of the steroid present in least quantity is about the same as for the steroid present in greatest quantity, the error expressed as a percentage of the absolute value is large for steroids present in small quantity (18). This, however, does not obscure the trend of the results.

Dextrose analysis.

The reducing power of the fermentation broth was considered to be a measure of the amount of dextrose that it contained. The

TABLE 1.

Composition of Nutrient Media Used.

	Medium 1*b* Natural g/l	Medium 2*c* Defined g/l
Dextrose	10.0	10.0
$NaNO_3$	—	3.0
NH_4NO_3	—	2.5
KH_2PO_4	—	1.0
KCl	—	0.5
$MgSO_4 . 7H_2O$	—	0.5
$FeSO_4 . 7H_2O$	—	0.08
$ZnSO_4 . 7H_2O$	—	0.05
$MnCl_2 . 4H_2O$	—	0.05
Edamin ¹)	20.0	—
Corn Steep Liquor	6.3	—
Antifoam ²)	0.6	0.6
pH before sterilizing ³)	5.2	5.2
pH after sterilizing	4.5–4.8	4.5–4.8

¹) Edamin is an enzymatic digest of lactalbumin obtained from Sheffield Chemical Co., New York, N.Y.

²) Dow Corning Silicone Antifoam B.

³) Adjusted with NaOH.

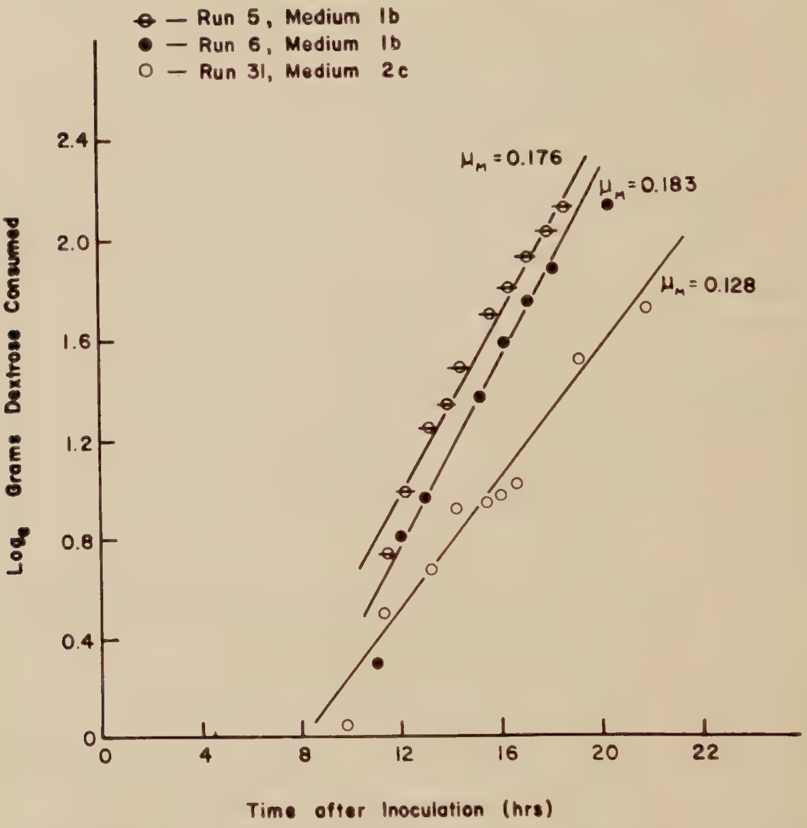

Fig. 2. Growth rate of *Aspergillus ochraceus* NRRL 405 in natural (1*b*) and defined (2*c*) media.

method used for the determination of dextrose was a modified Somogyi method (18) that was usable for the range 1—20 mg dextrose/aliquot. Therefore, amounts of dextrose less than 50 mg/l could not be accurately measured because the maximum volume of the aliquot analyzed was 20 ml.

Nutrient medium.

For the purpose of continuous fermentation, it is desirable to use a defined medium, so that there can be no question as to which nutrient is limiting. The medium used in this investigation was similar to that of Dulaney *et al.* (9), and dextrose was used as the limiting nutrient for growth. The micro-organism was carried on agar slants of the medium and was stored at 4°C. after several days incubation at 30°C.

The composition of the defined medium is presented in Table 1, along with the composition of a natural medium which has been used by previous workers (13, 24). Fig. 2 illustrates the maximum growth rates obtained on these media under the same conditions in a batch fermentation.

As it was more convenient to determine growth indirectly by the consumption of dextrose rather than directly by weighing the mycelium, it was necessary to determine the yield value, Y. Previous workers (12, 20, 21) have found that this yield value for any organism is constant over a wide range of growth rates, but tends to decrease at growth rates near the maximum. However, all the continuous fermentations were carried out at growth rates well under the maximum, so that the yield value should be a constant. The value for Y was determined by weighing the mycelium (dried *in vacuo* at 70°C. for 16 hours) produced by the consumption of a known amount of dextrose. The value of Y obtained was 0,471 ± .033 g mycelium/g dextrose consumed. This value was used throughout the investigation to convert dextrose consumption into mycelial concentration.

Sterilization and inoculation.

The nutrient medium reservoir, medium addition line, and hypodermic needle were sterilized as one unit. The fermentor, or fermentors if two stages were being used, and the air filter were sterilized as another unit. In general, the first-stage fermentor was sterilized containing about three liters of medium while the second-stage fermentor was sterilized empty. The sterilization was carried out in a vertical retort, and consisted of heating for 75 minutes at 258°F., followed by the introduction of cooling water while maintaining an air pressure of 15 p.s.i.g. in the retort to prevent boiling of the medium. It was found essential to bring the temperature of the medium below 140°F. within an hour after turning off the steam, or severe browning would take place with consequent development of growth-inhibiting substances. Asepsis during the fermentation was checked by differential plate counting with W-L differential medium (Difco Lab., Detroit) and microscopic examination.

The first-stage fermentor was inoculated with about 150 ml of a suspension of well-developed mycelium by means of a sterile funnel. If the mycelium showed any tendency to grow in a pellet rather than in a filamentous form, the inoculum was homogenized in a sterile Waring Blendor before inoculating. In a few instances, spores were used directly as inoculum, but this practice tended to result in subsequent pellet formation in the fermentor and so was discontinued.

RESULTS AND DISCUSSION.

Continuous fermentation.

The inoculum was allowed to develop until most of the dextrose in the fermentor had been used and then the continuous flow of nutrient medium was started. After a steady state had been reached, as measured by a constant dextrose concentration, progesterone addition to the first-stage fermentor was started. If the progesterone were being added to the second stage, addition would be started at the time effluent began to leave the second stage. After the addition of progesterone had been started, from 12—36 hours were allowed to elapse before samples were taken for steroid analysis. Then several samples would be taken over a period of 10—36 hours.

Fig. 3 presents data obtained during a typical continuous fermentation run. In the course of this investigation more than twenty continuous fermentations were carried out, and the pattern of dextrose concentration and pH presented in Fig. 3 was found to be typical of all the experimental runs. This pattern is different from that previously reported with bacteria and yeasts, where a steady state is easily achieved, and merits discussion.

It was observed with every continuous fermentation made with *A. ochraceus*, that the attainment of a steady state, if attained at all, proceeded in two phases. The inoculum grew, thus reducing the dextrose concentration and raising the pH. After the inoculum had consumed most of the dextrose present, a continuous flow of medium would be started, always using a dilution rate well under the maximum growth rate. For a while the dextrose concentration would continue to fall, sometimes reaching levels as low as those obtained in the true steady state (0.01—0.08 g/l).

This decrease in dextrose concentration, corresponding to an increase in micro-organism concentration, means that the micro-

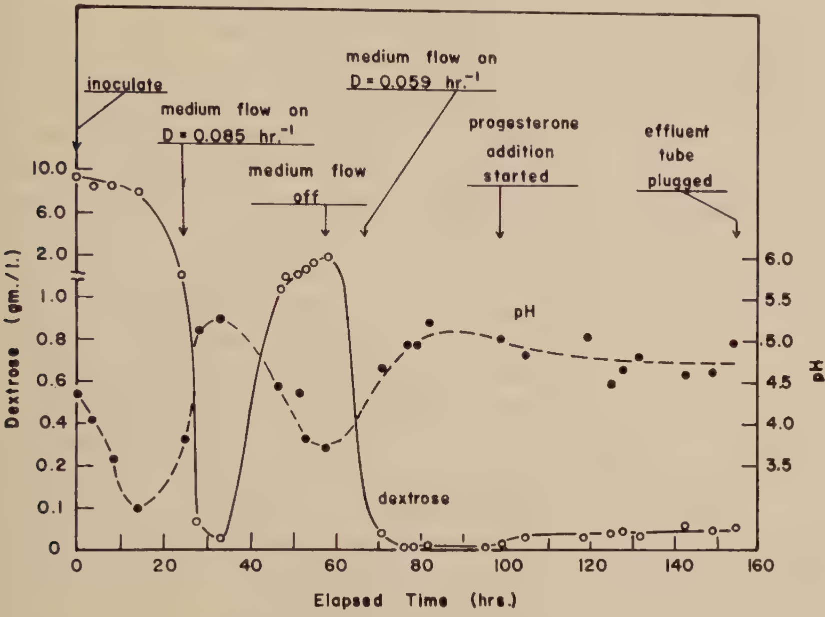

Fig. 3. Dextrose concentration and pH versus elapsed time for a typical continuous fermentation.

organism is growing more rapidly than it is being removed from the fermentor by the continuous flow of medium. This can be stated in mathematical terms as $\mu > D$.

According to theory, the concentration of dextrose should continue to fall, leading eventually to the steady state condition in which $\mu = D$.

However, at this point in the course of the fermentation the second phase began. Instead of settling into steady state operation, the dextrose concentration started to rise and the pH to fall. The situation had now changed and the growth rate was less than the dilution rate, or $\mu < D$.

If this trend continued the microorganism would be washed out. In a few cases, this washing-out process reversed itself without interference by the experimenter, but in the majority of successful continuous fermentations, it was necessary to reduce, or stop, the flow of nutrient medium for several hours to prevent wash out. In most cases a steady state was attained shortly after the flow of nutrient medium had been restarted, and in the few cases where it was not attained, the failure was due either to pellet formation because of insufficient mycelium in the fermentor or to plugging of an effluent tube.

The explanation for this two-phase phenomenon is not clear at the present time. It may be a form of microbial lag or overshoot of the type proposed by Finn and Wilson (10), but the phenomenon they reported does not appear to have been observed by other workers. Although a rise in pH was generally associated with a fall in dextrose concentration, and consequent achievement of a steady state, the pH itself does not appear to have been the cause of the change in growth rate because, when small quantities of sterile sodium hydroxide were added to the fermentor in an attempt to maintain the pH at a level consistent with the steady state, the pH of the medium very quickly reverted to its previous value, and the change in pH did not appear to influence either the growth rate or the future attainment of the steady state. In addition, this change in growth rate could not be attributed to a requirement for carbon dioxide, because the inocula were all actively growing at the time of inoculation, and furthermore, adding carbon dioxide to the air stream had no effect on the reversal of growth rate.

In a few continuous fermentations, the mycelium grew in the form of pellets rather than the desired filamentous form. This was found to be caused by an insufficient inoculum, or more often, by partial wash out of the mycelium in the fermentor. A low mycelial concentration has been found by previous workers (28) to encourage growth in the form of pellets, which were undesirable for two reasons: (i) the removal of pellets from the fermentor by the effluent tube was inefficient and resulted in the effective dilution rate for the microorganism being less than the dilution rate of the broth, and (ii) the mycelium was not homogeneous, in that the cells at the center of a pellet did not have the same supply of oxygen and other nutrients as the cells at the surface of a pellet, and therefore their metabolic activity and growth rates were different.

In general, steady state was attained 70—90 hours after inoculation, and the fermentations continued for another 40—90 hours before being terminated. The reason for termination in the large majority of cases was the plugging of the effluent tube by mycelium. Mechanical breakdowns, leaks in tubing, and contaminations were minor problems compared to the problem presented by the accumulation of mycelium within the first-stage fermentor and its effluent tube. Only one fermentation was terminated due to contamination. The extent of the difficulties caused by accumulation of mycelium has been described by Bartlett and Gerhardt (2), and an elegant solution to these problems, involving vortex aeration and choice of first-stage conditions to achieve more desirable morphological forms, has been presented by Pirt and Callow (25) for the case of the penicillin fermentation.

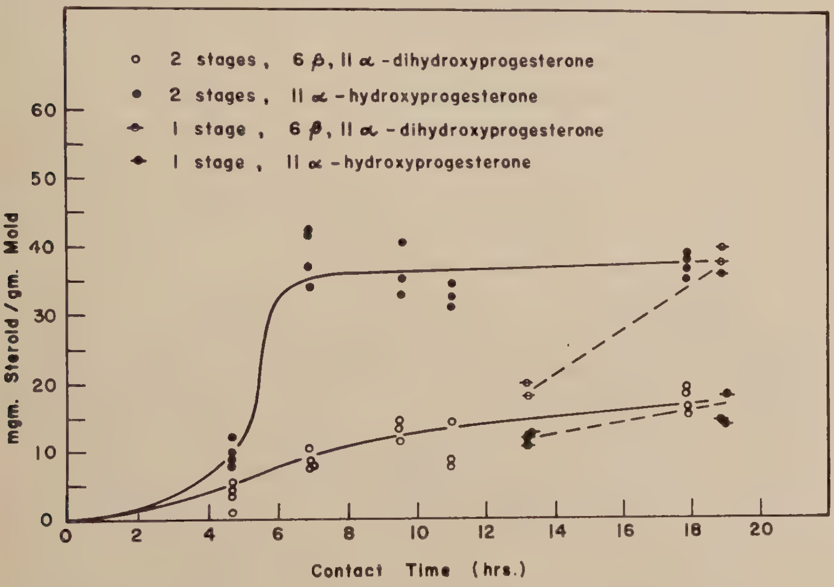

Fig. 4. Concentration of 11α-hydroxyprogesterone and 6β, 11α-dihydroxyprogesterone as a function of contact time for several different continuous fermentation runs. Each point represents the analysis of a single aliquot of broth. The aliquots were taken at 3–8 hours intervals during steady-state operation.

Progesterone conversion.

Fig. 4 presents a plot of concentration of 11α-hydroxyprogesterone versus the contact time, for both one and two-stage fermentations. Initially, one-stage fermentations were used (represented by the dashed lines), but the progesterone was found to interfere with the growth of the microorganism (5, 6, 15), and as a result the dilution rate could not be increased sufficiently to give a short contact time and a high yield of 11α-hydroxyprogesterone. Therefore continuous fermentations were made in two stages: The microorganism was grown in the first-stage fermentor, and the progesterone was added and hydroxylated in the second stage. The contact times were varied by changing either the medium flow rate or the liquid level in the second-stage fermentor.

It is clear that for a two-stage system the plot of concentration of 11 α-hydroxyprogesterone *versus* time is substantially different from that obtained in batch fermentations (9, 13) where it reaches a peak in 6—12 hours and then falls rapidly. The reason for this difference may be due to the fact that in the two-stage continuous fermentation, the microorganism enters the second stage and comes into contact with the progesterone after growth has taken place. Therefore there is no competition between protoplasm synthesis and adaptive enzyme synthesis. The shape of the 11α-hydroxyprogesterone curve appears to indicate that its formation is due to an autocatalytic reaction. This, however, can be interpreted to mean that the observed rate of hydroxylation is constant with respect to enzyme concentration, but that the enzyme concentration increases for the first six or seven hours of contact time after exposure to the progesterone. This long enzyme induction period has been observed in previous investigations (17, 23, 27) on the nature of microbial steroid hydroxylases. It appears that maximum enzyme concentration is reached in approximately six hours, and that then a steady state in the concentration of 11α-hydroxyprogesterone is reached, in which its rate of formation from progesterone is equal to its rate of hydroxylation to 6β, 11α-dihydroxyprogesterone.

The steady state in the concentration of 11α-hydroxyprogesterone which extends from contact times to 7—19 hours may possibly be explained by the following hypothesis:

Let N_a be the concentration of progesterone, N_b the concentration of 11α-hydroxyprogesterone, and N_c the concentration of 6β, 11α-dihydroxyprogesterone. If the hydroxylations proceed by a first order reaction mechanism, the following equations apply

$$-\frac{dN_a}{dt} = k_1 N_a \tag{1}$$

$$\frac{dN_b}{dt} = k_1 N_a - k_2 N_b \tag{2}$$

$$\frac{dN_c}{dt} = k_2 N_b \tag{3}$$

where k_1 and k_2 are the reaction rate constants. Considering only the period of contact time greater than 7 hours, it can be seen from Figure 4 that

$$\frac{dN_b}{dt} = 0$$

and

$$\frac{dN_c}{dt} = \text{constant} = k_2 N_b$$

Substituting these observed relationships into Equation 2,

$$0 = k_1 N_a - \text{constant} \tag{4}$$

or

$$k_1 N_a = \text{constant} \tag{5}$$

Substituting Equation 5 into Equation 1,

$$-\frac{dN_a}{dt} = k_1 N_a = \text{constant} \tag{6}$$

Equation 6 is self-contradictory, however, because although the concentration of progesterone is continually declining with increasing contact time, its rate of decline remains constant, rather than falling with decreasing concentration of progesterone. This contradiction can be resolved if it is assumed that the reactant is not progesterone *per se*, but some dissolved or activated form of progesterone. This is a logical assumption, because for an enzyme to act on a substrate at a measurable rate, the substrate must be in solution or a very fine state of division. Progesterone, however, is not very soluble in aqueous solutions, and when a solution of progesterone is added to an aqueous solution, a precipitate forms immediately. If $N_a{}^*$ is the concentration of dissolved progesterone, then

$$N_a \gg N_a{}^*$$

Therefore the concentration of dissolved progesterone, $N_a{}^*$, will remain constant as long as there is undissolved progesterone in suspension. Equation 1 should therefore be modified to read:

$$-\frac{dN_a}{dt} = k_1 N_a{}^* \tag{7}$$

Using the modified form, Equation 7, it is seen that the rate of hydroxylation of progesterone is constant as long as the concentration of dissolved progesterone is constant; a state which should exist until there is very little unconverted progesterone remaining in the fermentation broth. Apparently, even at contact times of 19 hours, the concentration of undissolved progesterone was high enough to maintain the concentration of dissolved progesterone at a constant level.

Although Fig. 4 indicates that the yield of 11α-hydroxyprogesterone in a two-stage system is constant at about 36 mg/g mycelium for contact times for 7—19 hours, if the data are presented so that rate of output of 11α-hydroxyprogesterone is plotted against the contact time (Fig. 5), it is seen that there is a relatively narrow optimum contact time for maximum rate of output of 11α-hydroxyprogesterone.

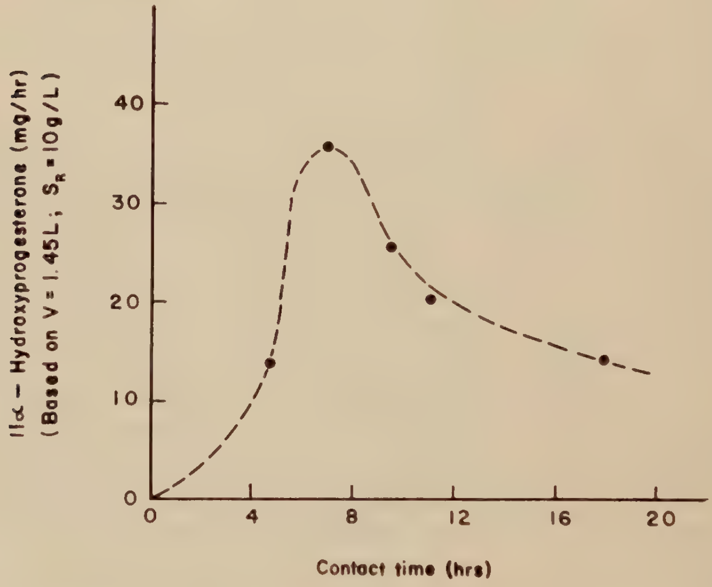

Fig. 5. Rate of output of 11α-hydroxyprogesterone as a function of contact time for a two-stage continuous fermentation with continuous steroid addition to the second stage. Second stage volume, 1.45 l.

Summary.

In this paper are presented the results of experiments undertaken to determine what conditions are necessary for the hydroxylation of progesterone during continuous culture of *Aspergillus ochraceus* NRRL 405. It was found possible to operate with either one or two stages, but because of the inhibitory effect of progesterone on the growth of the microorganism, optimum yields of 11α-hydroxyprogesterone could be obtained only with a two-stage system in which the organism was grown continuously in the first stage and progesterone was added continuously and hydroxylated in the second stage.

In addition it was observed that there was an apparent cycling of the microorganism concentration in the period preceding the steady state. No explanation for this was found, and this problem in dynamics is being investigated further.

Details are presented of an isotope dilution method using 4-C^{14}-progesterone for determining the concentration of progesterone and its derivatives in fermentation broths.

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n-ALKANE OXIDATION BY A *PSEUDOMONAS*

FORMATION AND β-OXIDATION OF INTERMEDIATE FATTY ACIDS

by

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(Received June 26, 1960).

INTRODUCTION.

In earlier work (THIJSSE and VAN DER LINDEN, 1958) it has been shown — with the aid of multiple adaptation techniques — that *n*-alkanes are oxidized by *Pseudomonas* 473 along the following pathway: *n*-alkane, primary alcohol, aldehyde, fatty acid (presumably as a co-enzyme A complex). Strong indications were found for the degradation of fatty acids by means of a β-oxidative mechanism whereas the occurrence of a second degradation mechanism, such as decarboxylation, seemed highly unlikely.

In view of contradictory opinions in the literature (see discussion) it was thought necessary to obtain unequivocal evidence for our previous results by isolating and identifying (*e.g.* by mass spectrometry) the key intermediates in the alkane degradation. Now, our previous investigation had shown that the respiratory quotient of resting *Pseudomonas* cells with heptane as a substrate is 0.62. Since the theoretical value for oxidation to carbon dioxide and water is 0.64, the presence of detectable amounts of intermediate alkane oxidation products in the culture medium is unlikely. It proved possible, however, to induce some accumulation of these products by a 25% decrease of the oxygen supply to the laboratory fermentor used, during the last few hours of growth. In these experiments heptane and hexane were used. They were the only carbon sources available to the micro-organism.

EXPERIMENTAL.

1. General data.

Micro-organism. The micro-organism used throughout these experiments is a *Pseudomonas* strain (KSLA no. 473) that has been described earlier (THIJSSE and VAN DER LINDEN, 1958); it is believed to be identical or closely related to *Ps. aeruginosa*.

Medium. The mineral medium used had the following composition:

KH_2PO_4	4.50 g	H_3BO_3	2.9	mg
Na_2HPO_4	3.75 ,,	$MnCl_2 . 4 aq$	1.8	,,
KNO_3	5.00 ,,	$ZnSO_4 . 7 aq$	0.22	,,
$MgSO_4$	0.25 ,,	$CuSO_4 . 5 aq$	0.08	,,
		$H_2MoO_4 . 1 aq$	0.015	,,
		$FeCl_3$	0.005	,,

De-ionized water up to 1 litre; pH 6.4

Heptane (ASTM grade) and hexane (pure grade) were commercial samples (Phillips). The heptane was redistilled and then found to be pure by gas-liquid chromatography. The hexane did not need redistillation. The alkanes were dosed as indicated in the following section.

Fermentor. The laboratory fermentor consisted mainly of a 1-metre Hysil glass column (Quickfit) having a volume of about 9 litres. Intake air was dispersed by a glass filter at the bottom. The column was kept in vibration by a pneumatic device during cultivation, since the air bubbles are then more easily released from the air dispersion filter. Hence, they remain smaller and their distribution in the culture medium is greatly improved, thus increasing the amount of oxygen available to the micro-organism.

By recycling the culture liquid from the bottom to the top of the column the efficiency of aeration was further improved.

The hydrocarbons were dosed by means of a burette, which was kept under the same pressure as the intake air. They were injected into the intake air stream and vaporized by it.

The apparatus was placed in a room of constant temperature (25°C.).

Cultivation procedure. After inoculation of the column with alkane-adapted *Pseudomonas* cells, the air supply was increased gradually so as to follow the growth of the micro-organism; *e.g.* 4 litres/hr on the first day, 6 litres/hr on the second, up to 10 litres/hr on the third or fourth. The oxygen uptake at the end of the culture period was approximately 11% calculated on air intake, that is more than 1 litre O_2/hr.

In order to induce some fatty-acid accumulation the air stream was reduced to 7—8 litres/hr, 4—5 hours before harvesting. The concentration of the bacteria during this period was about 10 g (wet weight) per litre.

2. Isolation and identification of fatty acids.

Isolation and esterification. Since the analysis of the total culture broth and that of the supernate yielded essentially the same information the micro-organisms were generally separated from the culture broth in a Sharples centrifuge.

Potassium hydroxide was added to the centrifugate ($pH > 10$) and the liquid was concentrated to 200 ml by distillation. Trioctyl phosphate (0.3 ml) was added to prevent foaming. After acidification with sulphuric acid, magnesium sulphate was added to saturation in order to facilitate subsequent steam distillation.

The distillate was extracted with ether and the ethereal solution concentrated (20 ml), dried over anhydrous sodium sulphate and treated with excess diazomethane (VOGEL, 1951) prepared from nitrosomethylurea. The methylester solution was concentrated (15 ml) by distilling off ether through a 2 m Vigreux column and then subjected to gas-liquid chromatography.

Gas liquid chromatography (GLC).¹) A 4 m column (stationary phase: polyethylene glycol 400) employing a katharometer as the detection system was operated at 100°C. The injection volume was 0.05—0.30 ml. The apparatus was calibrated with the aid of pure butyric, caproic and heptylic acids, which were esterified as described above. The chromatogram represented in Fig. 2 was obtained by use of a 6 m column.

Mass spectrometry²). If possible the methyl-ester fractions were collected separately from the GLC-column effluent gas by freezing and subsequently analysed by means of a mass spectrometer (consolidated model 21—103C) calibrated with appropriate model compounds.

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¹) Valuable advice from Dr. A. P. ZEELENBERG is gratefully acknowledged.

²) The authors thank Dr. C. LA LAU, Mr. W. G. VAN WIJK and Miss G. MARBUS for carrying out the mass-spectrometric analyses.

Results.

Heptane oxidation.

A mixture of fatty-acid methyl esters from one particular heptane oxidation experiment was analysed by GLC in triplicate. The results are shown in Fig. 1 and Table 1. Although the total amounts of fatty acids found may vary considerably when various fermentation fluids are analysed, the analysis presented is representative of both the low C_4 and C_6 values and the high C_5 and C_7 values. The C_3 fraction seems most variable when various fermentations are compared.

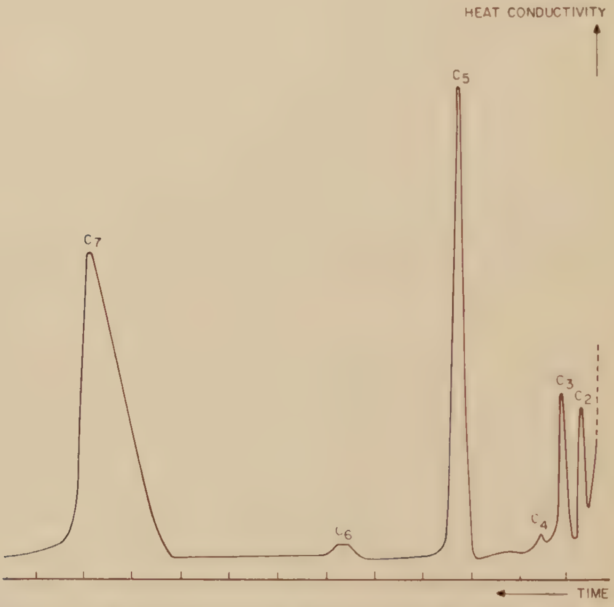

Fig. 1. Gas-liquid chromatogram of fatty acids (as methyl esters) formed in the bacterial oxidation of *n*-heptane.

By mass spectrometry additional information was obtained for various fractions: 55% of the C_3 fraction turned out to be methyl propionate. The residual 45% could not be identified owing to lack of pure model compounds. The C_5 fraction was identified as methyl *n*-valerate of 95% purity, whereas the C_7 fraction turned out to be methyl *n*-heptanoate of 99% purity. The C_6 fraction (mol.wt. 114) could not be identified but was definitely not identical with caproic-acid methyl ester. Mass-spectrometric evidence may indicate

TABLE 1.

GLC of fatty-acid methyl esters from a heptane oxidation experiment.

Methyl ester of	Integrated peak areas [1]				Amount	
	Anal.No.1	Anal.No.2 [2]	Anal.No.3	Mean	mg [3]	%
C_3		51.1	80.0	65	35	6.3
C_4		7.2	10.8	9	5	0.9
C_5	258.6	240.1	266.6	255	145	26.2
C_6	13.8	24.7	16.8	18	10	1.7 [4]
C_7	669.2	637.0	688.0	665	360	64.9
Total					555	100.0

[1] In arbitrary units, corrected for injection volume of 0.1 ml.

[2] Represented in Fig. 1.

[3] In mg free acid per litre of original culture, from which 3 litres were taken for analysis.

[4] If fraction C_6 contains methyl caproate, this amounts to max. 7% of this fraction.

a cyclic or an unsaturated fatty-acid methyl ester derived from a C_6 hydrocarbon. This indication, however, is not in line with the chromatographic behaviour of the C_6 fraction. The C_4 fraction was not investigated by mass spectrometry because of its small amount.

The C_2 fraction could not be separately collected by GLC. Its retention volume indicates that it is probably not identical with methyl acetate.

Hexane oxidation.

Although the total amounts of fatty acids formed in hexane oxidation experiments varied notably, the fatty-acid composition was fairly constant, particularly with respect to propionic acid, the amount of which was invariably low.

The GLC-analysis of one typical experiment is summarized in Fig. 2 and Table 2.

The results show that the C_5 fraction was absent. The C_2, C_3 and C_4 fractions were too small for mass-spectrometric identification. The retention volumes indicated that they are identical with acetic, propionic and butyric-acid methyl esters. If they are, they may well be common cell metabolites not directly related to hexane degradation. The C_6 fraction was identified by mass-spectrometry as methyl caproate of 99% purity.

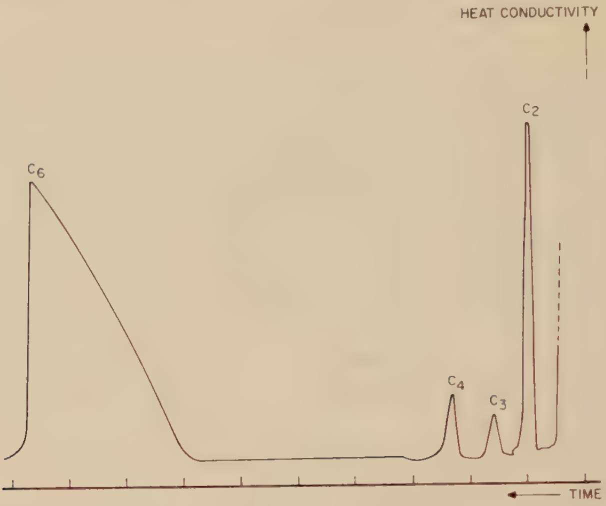

Fig. 2. Gas-liquid chromatogram of fatty acids (as methyl esters) formed in the bacterial oxidation of *n*-hexane.

TABLE 2.

GLC of fatty-acid methyl esters from a hexane oxidation experiment.

Methyl ester of	Integrated peak area [1])	Amount	
		mg[2])	%
C_2	27.5	1.9	0.9
C_3	10.0	0.7	0.3
C_4	17.4	1.1	0.6
C_5	0.0	0.0	0.0
C_6	3075.0	204.5	98.2
Total		208.2	100.0

[1]) In arbitrary units; injection volume 0.3 ml; methyl ester concentrate 15 ml.

[2]) In mg free acid per litre of original culture, from which 8 litres were used for analysis.

DISCUSSION.

Identification of heptylic, valeric and propionic acids clearly demonstrates primary attack on C_1 of the heptane molecule as well as β-oxidation of intermediate fatty acids. If caproic acid occurred at all in the heptane culture liquid its concentration was less than 0.2% of the heptylic acid concentration. Consequently, degradation by decarboxylation — *e.g.* such as has been found in peanut cotyledons (STUMPF, 1956) or non-enzymatic decarboxylation (GEYER *et al.*, 1955) — is absent.

In hexane oxidation experiments the formation of caproic acid could be demonstrated conclusively, whilst here valeric acid was shown to be absent. This experiment thus confirms primary attack on C_1 as well as the absence of oxidative decarboxylation.

In 1955 TRECCANI postulated — on the basis of multiple-adaptation experiments — that a C_1 breakdown mechanism occurs in addition to β-oxidation in the metabolism of alkanes from C_6 upwards. In an earlier paper we have already shown by extension of TRECCANI's experiments that his work provides no evidence of a decarboxylation reaction. From our present results it may safely be concluded that this reaction indeed does not occur in our *Pseudomonas* strain.

SENEZ *et al.* (1956) originally favoured C_1 breakdown, whilst they rejected β-oxidation. Their results were based on identification of fatty acids by means of paper chromatography. The results of multiple-adaptation techniques, however, have recently led AZOULAY and SENEZ (1960) to support the exclusive operation of a β-oxidation mechanism.

Although the procedure used in the present investigation is not suitable for isolation of dicarboxylic acids, there is as yet no reason to assume an additional degradation system attacking both ends of the molecule. In fact we have shown earlier (THIJSSE and VAN DER LINDEN, 1958) that the C_6-dicarboxylic acid (adipic acid) is not metabolized by hexane-adapted *Pseudomonas* cells. This has now been confirmed by AZOULAY and SENEZ (1960), who used pimelic acid and heptane-grown cells.

No more does our investigation provide detailed information on the reactions occurring in the β-oxidation cycle of fatty acids. However, GOLDMAN and GELBACH (1959) recently showed that the same sequence of reactions obtains in aerobic bacteria as has been found in animal tissue.

Summary.

From a culture broth of *Pseudomonas aeruginosa* (KSLA strain 473) grown on heptane as the sole source of carbon, fatty acids could be isolated after a period of decreased oxygen supply. The

corresponding methyl esters — obtained by treatment with diazomethane — were separated by gas-liquid chromatography and identified by mass spectrometry. Heptylic, valeric and propionic acids were shown to be present in the original culture broth.

Using the same techniques the formation of caproic acid from hexane was shown to occur, whereas the amount of butyric acid formed was extremely small and inconsistent.

These results show conclusively that this microbiological oxidation of heptane and hexane proceeds by way of the corresponding fatty acids, which are further degraded by β-oxidation. The absence of caproic and valeric acids in heptane and hexane oxidation, respectively, shows that decarboxylation of fatty acids does not occur.

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TAXONOMY OF *CRYPTOCOCCUS NEOFORMANS* AND ITS VARIETY *UNIGUTTULATUS*

by

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(Received September 12, 1960).

The yeast *Cryptococcus neoformans* has been the subject of studies from various points of view. Since it is known to be pathogenic for man it is of particular importance to have it well-defined. Taxonomic data are given by LODDER and KREGER-VAN RIJ (1952). The species is characterized as follows:

"Cells are round or almost round, 3.5—8 μ in diameter. They are surrounded by a capsule. Under appropriate conditions "starch" is formed in the capsule or in the medium. The appearance of the streak culture is mucous. No pseudomycelium formation. No ascospores. Fermentation absent. Assimilation of glucose, galactose, saccharose and maltose. Lactose and nitrate cannot be assimilated. Splitting of arbutin: slightly positive."

A variety *uniguttulatus* to the species was recognized for those strains in which the formation of a capsule is very reduced, almost to nought. The appearance of the streak culture on malt agar is not mucous. The type strain of the variety was originally described by WOLFRAM and ZACH (1934) as *Eutorulopsis uniguttulata*. This strain, as well as a second one classified in the variety by LODDER and KREGER-VAN RIJ, has been isolated from human diseased nails.

Cr. neoformans and its variety *uniguttulatus* may be distinguished from most other species in the genus *Cryptococcus* by the assimilation reactions with galactose, saccharose, maltose, lactose and nitrate. From the species *Cr. luteolus* which has the same assimilation pattern, it differs in the shape of the cells. In *Cr. neoformans* the cells are round to short-oval, in *Cr. luteolus* they are oval or long-oval to elongate, (3.5—6) × (6—11.5) μ. The original strain of the latter species does not grow at 37°C.

In a publication on the genus *Cryptococcus*, BENHAM (1955) mentions the variety *innocuous* as a non-virulent form of the species *Cr. neoformans*. The properties given are: no growth at 37°C.; not pathogenic for mice; assimilation of glucose, saccharose and maltose, and of nitrate. A strain of this variety from BENHAM's collection received by the courtesy of Dr. M. SILVA appeared to be able to assimilate glucose, galactose, saccharose, maltose and lactose, and could therefore be identified with *Cr. albidus*. The assimilation of galactose and lactose is often weak in this species.

VAN UDEN, BRAÇO FORTE and DO CARMO SOUSA (1959) isolated a strain of *Cr. neoformans* from the stomach of a horse and studied it together with 22 other strains of this species. They found as the maximum temperature for growth for all strains 39—40°C. Glucose, galactose, saccharose, maltose, *D*-xylose, mannitol, sorbitol and inositol could be assimilated. Raffinose, *L*-arabinose, ethanol and soluble starch were assimilated by most strains. Glycerol and inulin were weakly or not assimilated. Lactose and nitrate could not be utilized.

In publications of medical origin the ability to grow at 37°C. is generally mentioned as an important characteristic of *Cr. neoformans*. Out of the 26 strains of this species recorded by LODDER and KREGER-VAN RIJ (1952) 25 grow at this temperature. The strain which makes the exception (CBS. 963) was isolated from the air. The two strains included in the variety do not grow at 37°C.

In the last few years we have investigated strains of *Cr. neoformans* which were not, or only slightly mucous, and which grew at 37°C. Moreover, from one source of isolation more and less mucous strains were obtained all growing at 37°C. The question arose whether these non-mucous forms could be identified with the type strain of the variety *uniguttulatus* which does not grow at 37°C., in other words, whether a reduced capsule was in this case a suitable characteristic for separating the variety from the species. To find an answer to this question capsule formation, growth at 37°C. and the assimilation of a greater number of carbon compounds were studied in strains of the species and the variety.

MATERIAL AND METHODS.

33 strains were studied. All of them corresponded with the standard

description of *Cr. neoformans* and of the variety *uniguttulatus* given by LODDER and KREGER-VAN RIJ (1952). They are listed in Table 1.

For the assimilation tests the auxanographic method was used in the first instance. The compounds which gave a negative result were re-examined by means of the liquid medium test according to WICKERHAM and BURTON (1948).

RESULTS.

In 7 strains growing at 37°C. and 5 strains not growing at this temperature the assimilation of 25 carbon compounds was tested. In all strains positive results were found for: glucose, galactose, saccharose, maltose, *L*-sorbose, trehalose, melezitose, *D*-xylose, *L*-arabinose, *D*-rhamnose, adonitol, mannitol, sorbitol, α-methyl glucoside, salicin and succinic acid. None of the strains could utilize lactose and melibiose. Varying results were obtained for: cellobiose, raffinose, *D*-arabinose, *D*-ribose, erythritol, dulcitol and citric acid. The latter 7 compounds were used to compare 22 other strains. The results of the tests are given in Table 1.

From this appears that there is a marked difference in the assimilation reactions between the strains growing at 37°C. and those which do not grow at this temperature. This applies especially to the use of dulcitol and *D*-arabinose. To a lesser extent it is the case with cellobiose, *D*-ribose and citric acid; for erythritol and raffinose it does not hold. In all instances when dulcitol was utilized, it could be observed in the auxanogram; *D*-arabinose generally gave a positive result only in the liquid medium test.

It was mentioned before that also non-mucous strains were found to grow at 37°C. This was the case with strain CBS. 4572, isolated from spinal fluid. In other strains young malt agar cultures were less mucous than older ones.

The capsules of the strains not growing at 37°C. are all narrow. The cells of these strains are short-oval rather than round. The consistency of the cultures is thready.

Strain CBS. 963 is in several respects an exception. Unlike the other strains tested it can use melibiose. While it does not grow at 37°C. it is very mucous and has round cells. Table 1 shows that it assimilates cellobiose, *D*-ribose and citric acid in contradistinction to the other strains of the second group.

TABLE 1.

Growth at 37°C. and assimilation of a number of carbon compounds by strains of *Cr. neoformans* and th variety *uniguttulatus*.

Strain CBS. No.	37°C.	dulcitol	*D*-arabinose	cellobiose	*D*-ribose	erythritol	raffinose	citric acid	Sender of the strain and year of receipt	Further details
132	+	+	+	+	+	w +	—	+	GIORDANO, 1935	Probably the original strain of SANFELICE.
131	+	+	+	+	+	+	—	+	"Institut Pasteur", 1912	
939	+	+	+	+	+	+	—	+	POLLACCI, 1930	
881	+	+	+	+	+	w +	+	+	BENEDEK, 1929	
879	+	+	+	+	+	+	+	+	OTA, 1924	
880	+	+	+	w +	+	+	—	+	VOSS, 1925	
885	+	+	+	+	+	+	—	+	WEIDMAN, 1933	
919	+	+	+	+	+	—	+	+	WEIDMAN, 1933	Isolated by SHEPPE.
884	+	+	+	+	+	+	+	+	BAUDET, 1934	
950	+	+	+	+	+	—	—	+	BIERBERG, 1910	*Saccharomyces* spec. Plimmer
464	+	+	+	+	+	+	—	+	NEGRONI, 1947	*Saccharomyces blanchardi.*
838	+	+	+	+	+	+	+	+	CASTELLANI, 1935	*Torulopsis hominis* var. *honduriana*, isolated from blastomycosis of the skin.
996	+	+	+	+	+	—	+	—	NIÑO, 1936	*Cryptococcus psicrophylicus* isolated from generalized blastomycosis.
1143	+	+	+	+	+	+	+	+	TODD, 1936	Isolated from spinal fluid.
1622	+	+	+	+	+	—	+	+	A.T.C.C., 1939	*Saccharomyces* spec. Curtis
1009	+	+	+	+	+	+	+	±	STENVERS, 1936	Isolated from the centra nervous system.
917	+	+	+	+	+	+	—	+	SEILER, 1937	Isolated from a case of blastomycosis.
882	+	+	+	+	+	+	+	+	N.C.T.C., 1932	*Torula nasalis*, isolated from a nasal tumor of a hors
918	+	+	+	+	w +	w +	+	+	WOLFF, 1949	Isolated from a white mous
1930	+	+	+	+	+	—	+	+	KRANEVELD, 1953	Isolated from a sick goat on Aruba.
1931	+	+	+	+	+	+	+	+	EMMONS, 1952	Isolated from a mouse afte inoculating it with a so sample (EMMONS, 1951).
1933	+	+	+	w +	+	—	+	+	EMMONS, 1952	Isolated from a case of mastitis in a cow.
2502	+	+	+	+	+	+	+	+	BEUTE, 1956	Isolated from the spinal flui in a case of meningitis.
4195	+	+	+	+	+	+	+	+	HOLTZ, 1958	Isolated from a case of lymphogranulomatosis.
4572	+	+	+	+	+	+	+	+	TORHEIM, 1960	Isolated from the spinal flui in a case of meningitis.
1935	+	+	+	+	+	+	+	—	EMMONS, 1952	Isolated from soil *via* a mouse.
4194	+	+	+	+	+	+	+	+	HOLTZ, 1958	Isolated from a case of lymphogranulomatosis.

w — weak.

TABLE 1 (*continued*).

train BS. No.	37°C.	dulcitol	*D*-arabinose	cellobiose	*D*-ribose	erythritol	raffinose	citric acid	Sender of the strain and year of receipt	Further details
730	—	—	—	—	±	—	+	—	Zach, 1935	*Eutorulopsis uniguttalata*, from human diseased nails.
727	—	—	—	—	—	—	+	—	Prakken, 1950	Isolated from human diseased nails.
257	—	—	—	—	—	—	+	—	Mackenzie, 1959	Isolated from sputum.
770	—	—	—	—	—	—	+	—	Beute, 1956	Isolated from the throat.
994	—	—	—	—	—	—	+	—	Cochet, 1957	Isolated from the bronchus of a patient with pulmonary disease.
963	—	—	—	+	+	—	+	+	Winter, 1924	Isolated from the air.

Discussion.

Three properties of the species *Cr. neoformans* and its variety *uniguttulatus* have been studied: capsule formation, ability to grow at 37°C., and a number of assimilation reactions.

From a taxonomic point of view it seems reasonable to consider a non-capsulated form of a capsulated yeast as a variant of the species, provided that they agree in all other respects.

From a medical view-point the characteristic of growth at 37°C. is of great practical importance. It is not probable that a yeast which fails to grow at 37°C. will cause internal lesions. Perhaps this does not hold for superficial infections, since two of the *uniguttulatus* strains came from diseased nails.

A difference in assimilation reactions may be used to differentiate species. A differentiation based on more than one compound, especially in the case of the non-fermenting yeasts, seems preferable. It is also advisable to study a great number of strains of both types to see whether the differences between them are general. In the case of the var. *uniguttulatus* the number of strains studied was relatively small.

It was found that the non-mucous form of *Cr. neoformans* coincides in many cases with absence of growth at 37°C. and the inability to assimilate some particular carbon compounds. However, there are also non-mucous forms which do grow at 37°C. and have the same assimilation pattern as the mucous ones. Thus not all non-mucous strains correspond in assimilation reactions and temperature of growth with the type strain of the var. *uniguttulatus*. The difference in assimilation reactions seems to us of more importance for the differentiation of the variety from the species than capsule formation. Moreover the assimilation of certain compounds correlates with growth at 37°C. Therefore we propose for this differentiation to replace capsule formation by assimilation reactions and temperature of growth. The classification of the variety *uniguttulatus* as a separate species might be considered. However, for the present we prefer to retain it as a variety.

The species *Cr. neoformans* may be characterized as follows: Cells are round or almost round, 3.5—8 μ. They are generally surrounded by a capsule which may, however, be very reduced. Under appropriate conditions "starch" is formed in the capsule. The appearance of the streak culture is mucous or not. No pseudomycelium formation. No ascospores. Fermentation absent. Assimilation of glucose, galactose, saccharose, maltose, dulcitol and *D*-arabinose. Lactose and nitrate cannot be used. Arbutin is split. Growth at 37°C.

The variety *uniguttulatus* differs from the species in the following properties: Cells are round to short-oval, (3 — 7) × (4 — 9.5) μ. No assimilation of dulcitol and *D*-arabinose. No growth at 37°C.

Summary.

A comparative study of a number of strains of the species *Cr. neoformans* and its variety *uniguttulatus* showed that capsule formation was inappropriate as a character to differentiate species and variety. The ability to grow at 37°C. and to use dulcitol and *D*-arabinose as source of carbon appeared to be more suitable. A definition of species and variety on this basis was given.

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THE TYPE CULTURE CONCEPT IN *ACETOBACTER* AND OTHER GENERA

by

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DEFINITIONS.

"Mutation" and "Mutant". These are used, as previously, in the widest possible sense, to refer to any heritable change in the properties of a bacterium, whether this may involve genes, plasmagenes, recombination, transduction, and so on.

"Over-oxidising". This means that ethanol is first oxidised to acetic acid, and then the latter is "over-oxidised" to CO_2 and H_2O.

"Hoyer's Medium". For brevity this is used to refer to Hoyer-Frateur medium (FRATEUR, 1950), which is of the following composition: 1 g $(NH_4)_2SO_4$; 0.1 g K_2HPO_4; 0.9 g KH_2PO_4; 0.25 g $MgSO_4$; 30 ml ethanol. Make up to 1 litre with distilled water, and add 0.5 ml of a 1% solution of $FeCl_3$.

"*Acetobacter*". This is used as re-defined by LEIFSON (1954) to include only over-oxidising acetic acid bacteria. These, if motile, have peritrichous flagella. *Acetomonas* Leifson is excluded.

I. The Type Species of *Acetobacter*.

In the International Bacteriological Code of Nomenclature (BUCHANAN, ST. JOHN-BROOKS, and BREED, 1948) it is laid down, under Principle 11, that "The type of a generic name is a species, that of the name of a species or sub-species (variety) is usually an authentic culture, a specimen, or a preparation. In some species, however, the type is a description or a figure by a previous author".

Although this definition admits the use of the Linnaean concept of a species (*i.e.* a description) taxonomic authority usually emphasises the necessity for comparison with an actual culture of the authentic strain concerned. Thus Prof. R. E. BUCHANAN has recently written (private communication) "*Acetobacter* is the name of that genus of bacteria which, in the view of a competent bacteriologist, have a sufficient number of characters in common with the type species. A species in bacteriology is the type culture together with other strains found to have a sufficient number of characters in common with the type strain or culture".

These definitions, as voiced by Prof. BUCHANAN, and apparently shared by the majority of taxonomists, seem to raise, however, two questions. Firstly, what is the definition of a "competent" bacteriologist? Secondly, what is the definition of a "sufficient number"? In *Acetobacter* this latter point is of the utmost importance, for the species *A. paradoxum* has only one of FRATEUR's (1950) positive biochemical criteria in common with the alleged "type species" of the genus ("*A. aceti*") as will be seen from Table 1. But that one property is nothing less than the oxidation of ethanol to acetic acid and thence to CO_2 and H_2O. And this, despite what neo-Adansonians may contend, defines the genus *Acetobacter*.

TABLE 1.

Properties of *A. aceti* and *A. paradoxum*

Organism	"*A. aceti*"	*A. paradoxum*
Ethanol to acetic acid and thence to CO_2 and H_2O	+	+
Catalase	+	—
Growth on Hoyer's medium	+	—
Dihydroxyacetone from glycerol	+	—
Gluconic acid from glucose	+	—
Cellulose production	—	—

If it is argued that one cannot establish a genus merely on the basis of one property, then the genus *Acetobacter* must disappear, and although *A. paradoxum* would not then be an *Acetobacter* species, it would still be an "over-oxidising" acetic acid bacterium, and, moreover, one of the commonest ones encountered in wine (DUPUY, 1957). Furthermore there are other genera, and even families, which are virtually established by their possessing some single outstanding property, *e.g.* lactic acid-production in *Lactobacillus.*

It has previously been shown (SHIMWELL, 1958) that nearly all of the many strains of *Acetobacter* examined so readily gave rise to mutants indistinguishable from previously named classical *Acetobacter* species, or constituting "new species", that it seems virtually impossible to classify *Acetobacter* strains, no matter what criteria are used. However, it is probable that, in the next edition of BERGEY'S Manual, another attempt will be made to do this, on orthodox lines, and it is the purpose of this paper to assess the prospects of classifying *Acetobacter* species according to the Linnaean concept supplemented by the type culture concept, or by either separately if necessary.

In the 7th edition of BERGEY'S Manual (1957) the type species of *Acetobacter* is given as "*Acetobacter aceti* (Beijerinck, 1898) Beijerinck, 1900". In the opinion of FRATEUR (1950) however (with which we concur) "*A. aceti*" should primarily be attributed to PASTEUR (1864) who was the first to show that a micro-organism, which he called *Mycoderma aceti*, was responsible for the oxidation of ethanol to acetic acid in vinegar making. The name should surely therefore be *Acetobacter aceti* (Pasteur) Beijerinck, although there also seems some doubt whether BEIJERINCK ever bothered to publish "validly" the generic name *Acetobacter* either.

However, the point at issue is that *A. aceti* is almost everywhere recognised as the type species of *Acetobacter.* Consequently it is this species of which the authentic strain and/or original description must, at present, be used for the purpose of classifying other isolates if the type concept and/or the Linnaean concept are/is to be used.

Unfortunately, however, there is no authentic culture of the original strain extant, as PASTEUR recorded his discovery before the days of solidified media and did not isolate the organism. Nor, for the same reason, is there any "adequate" original description. Virtually all PASTEUR said about this organism, biochemically, was that it oxidised ethanol to acetic acid and thence to CO_2 and H_2O — which, presumably, would be considered too meagre a diagnosis by modern taxonomic standards. Thus scarcely the Linnaean, and certainly not the type culture concept, can be used for defining

Acetobacter, if *A. aceti* should continue to be recognised as the type species.

Frateur (1950) it is true, has described later isolates which he believes may well be identical with Pasteur's organism, and which he consequently named *A. aceti.* He has based this belief largely on the fact that his strains would grow with ammonium salts as sole nitrogen source with ethanol as sole carbon source — a test, however, which Pasteur did not apply to *Mycoderma aceti*; Pasteur's mineral medium contained acetic acid in addition to the ethanol. Dupuy (1957) has also pointed out that Pasteur's organism was found in wine vinegar, whilst those of Frateur were chiefly isolated from "spirit" (alcohol) vinegar, where ammonium salts are the main nitrogenous nutriment. Dupuy has been unable to isolate from wine any acetobacters with the properties of Frateur's *A. aceti.* He therefore considers that *A. aceti,* as described by Frateur, should be regarded as a new species attributable to Frateur himself, and that the original "*Mycoderma aceti* Pasteur" may well have more closely resembled *A. mesoxydans* Frateur. Certainly the latter species is very commonly found in acetifying wine and beer, where the nitrogenous nutriment is organic.

If it be thought then, that *A. aceti* (Pasteur) Beijerinck is impossible to use as type species of the genus, one must ask which species should be regarded as the type. Should one adopt Frateur's conception of "*A. aceti*" as a neotype of Pasteur's organism, or should one look for another classical *Acetobacter* species of more weighty chronological priority? If the latter alternative be preferred, one must seek, presumably, the first one isolated and described after Pasteur's publication. But to fulfil the present-day attributes required of a "type species", it should have been adequately described originally, and/or the authentic strain still available.

A. pasteurianus (*Mycoderma pasteurianum* Hansen, 1879) seems, chronologically, the next choice, but again no authentic strain appears to exist today. Moreover, its important, interesting (and apparently unique) property of producing voluminous extra-cellular starch, recorded by Hansen and possessed by more recent isolates of presumably somewhat similar strains, has been found to be lost on laboratory media in the course of quite short maintenance periods, probably by irreversible mutation (Shimwell, 1957). Also Hansen's original description might scarcely be regarded as "adequate" by modern taxonomists.

Next on the list appears to be *Acetobacter xylinum* (Brown) Holland, isolated by ADRIAN BROWN in 1886. Although no authentic cultures of BROWN's strain appear to exist today, innumerable subsequent similar isolates have been obtained, all of which have possessed the same striking (and probably unique) property of producing thick, cellulosic, leathery pellicles on liquid media. Numerous strains have been deposited in collections, some with specific or varietal names different from, or additional to, "*A. xylinum*" respectively.

With the exception of one catalase-negative strain (*A. xylinum* var. *maltovorans* N.C.I.B. 8623) most of the others which we have examined have differed from FRATEUR's conception of "*A. aceti*" only in their inability to use ethanol as sole carbon source, and in possessing the power to produce voluminous, leathery, cellulosic pellicles. Furthermore, most of such strains have also been highly mutable, and, by a succession of biochemical loss-mutations, have given rise to four other classical species (*A. mesoxydans*, *A. rancens*, *A. ascendens*, and *A. paradoxum* some of which, in turn, have given rise to other "new" species with novel combinations of FRATEUR's criteria (SHIMWELL, 1958)). As most *Acetobacter* species also show a similar mutability (for example some *A. mesoxydans* strains can gain cellulose production to yield *A. xylinum*) *A. xylinum* can, paradoxically perhaps, be regarded as the most "typical" of the classical acetobacters, on account of its mutability.

It is important to point out, however, at this juncture, that neither *A. xylinum*, nor any of its mutants, has ever gained, in our hands, the ability to use ethanol as sole carbon source *i.e.* they have never grown on Hoyer's medium. Conversely, FRATEUR's "*A. aceti*" and the other Hoyer-positive species (*A. peroxydans*, *A. estunense*, and *A. lovaniense*) have never lost this property. Thus one can, in a way, divide the acetobacters into two groups, Hoyer-positive and Hoyer-negative. The Hoyer-positive species do freely give rise to a number of colony mutants, however, which yield cultures with different cultural characteristics, but biochemically (in respect of FRATEUR's criteria only) they have proved remarkably stable, in sharp contradistinction to the Hoyer-negative group in which biochemical mutation is usually so common that most cultures are, or soon become, mixtures of cells with different biochemical characteristics.

In selecting a type species for *Acetobacter* one is thus faced with paradoxical alternatives. Firstly, one can choose, say, *A. xylinum*, to represent the Hoyer-negative group; then it will be "typical" of that group in so far that according to the conditions of cultivation it will probably turn into some other Hoyer-negative species (or more probably into a mixture of several). Secondly, we can choose, say, one of FRATEUR's strains of *A. aceti*, and arbitrarily decide that it shall be regarded as a neotype of PASTEUR's organism. If one should do this, however, it will not be representative of most strains of *Acetobacter*, as the majority of these are stably Hoyer-negative.

However, it cannot be too strongly emphasised that whatever method may be adopted for the forthcoming classification of *Acetobacter* species, the fact must be faced that one will not be classifying "pure" cultures, but unstable mixtures of "species", the constituent cells of which are similarly unstable, the majority mutating biochemically, and the remainder culturally, and sometimes also morphologically.

Furthermore, if one changes the medium, in either its chemical composition or its physical state (as is necessary for the so-called "characterisation" of species) one will be almost certain to be changing the cell composition of the culture, so that a species different from the original isolate may soon outgrow the latter (SHIMWELL and CARR, 1960). This being the case, it is difficult to see how either the Linnaean concept or the type culture concept can be applied, except by making numerous drastic concessions to the actual facts.

If the latter alternative be considered permissible, one could adopt *A. xylinum* as the type species of *Acetobacter*, using only the Linnaean concept based on BROWN's (1886) original description, and discarding the type culture concept because (a) there is probably no type culture of BROWN's strain in existence, and (b) even if there were, it might well have mutated out of all recognition by now, in the course of being sub-cultivated for 75 years.

The Linnaean concept cannot be adopted in the case of many bacterial species described before the turn of the century, as such descriptions are often too meagre to be of use. BROWN's paper, however, is beautifully written, clear, concise, convincing and detailed. Although published in 1886 it could not possibly be described as "inadequate". Indeed, it contains much more information than does the description of *A. xylinum* in BERGEY's Manual (1957). Furthermore the latter description omits the majority of the important characters (notably biochemical) described by BROWN, and replaces these with different information obtained, not with BROWN's strain, but with later isolates.

Herein lies the danger of adding to, or substituting for, a pioneer's original description, numerous alleged biochemical and other properties not recorded for the authentic strain (and quite possibly not possessed by it).

Whether the suggestion that *A. xylinum* (one of the most mutable of all acetobacters) might be adopted as type species of the genus is likely to meet with approval we do not know; it is certainly not the first species to be described, and priority of publication seems to be one of the main reasons for the adoption of type-species throughout BERGEY's Manual.

If it is now contrary to judicial taxonomic policy to adopt *A. xylinum*, despite Principle 11 (BUCHANAN *et al.*, 1948) then there is one paradoxical advantage in retaining PASTEUR's *Mycoderma aceti* as type-species of *Acetobacter* — not merely in spite of there being no type culture nor Linnaean description, but because of those very facts, which immediately remove all the difficulties recorded in the previous part of this paper.

As there is no type culture one cannot be misled by its having probably mutated unrecognisably in the course of 96 years' maintenance. As there is little more description other than the one fact that it oxidises ethanol through acetic acid to CO_2 and H_2O, the anomalies consequent on rampant mutation of *Acetobacter* species in respect of other properties, and the taxonomic difficulties appertaining thereto (as described above) automatically disappear. Moreover all those species and strains of bacteria which comply with PASTEUR's single criterion can now be included in *Acetobacter*. Even *A. paradoxum* Frateur (1950) which, as previously mentioned, has only one property in common with FRATEUR's conception of *A. aceti*, fits as snugly into *Acetobacter* as does any other species, and does not risk being relegated to some vaguely defined genus such as *Achromobacter*, which might happen if one were to adopt Adansonian Theory, or to maintain, as some do, that the fact of its being a commonly occurring "over-oxidising" acetic acid bacterium is insufficient to justify its inclusion in *Acetobacter*.

II. OTHER GENERA.

It might be thought that when one came to consider other genera the picture presented by *Acetobacter* would prove to be unique, and that in such other genera the type-culture concept, virtually insisted on nowadays, might prove applicable. But on looking at the type species designated for various genera in BERGEY's Manual (1957) one finds, at the outset, that their selection has again largely been governed by priority of publication, and that many of them date back to well before the turn of the century. So it seems reasonable to expect that, as with *A. aceti*, one may find absence of authentic original strains and inadequacy of original descriptions.

That this is indeed the case has been pointed out by SHEWAN, FLOODGATE and HAYES (1958) who, when discussing the establishment of a National Type Collection of Marine Bacteria, write, "A primary handicap has been the disappearance of the type species of many of the genera commonly occurring in marine, soil, air or fresh water environments". In other words there are now only relatively few authentic cultures of the type species of the majority of saprophytic bacteria. SHEWAN and his co-workers go on to say, moreover, that "The description of these organisms in the literature as well as those of many related species are completely inadequate". It logically follows that neither the Linnaean nor the type-culture concept can be applied to many, and perhaps most, saprophytic bacteria.

Even if this were not the case, however, and if original type cultures were still to exist, there would still be the question whether, in the course of time, they would have undergone mutation to such an extent as no longer to be in accordance with their original descriptions. This raises the question whether cultures of other genera contain "mutants" to anything like the extent of that found in *Acetobacter*.

A preliminary assessment of this possibility has recently been made (SHIMWELL and CARR, 1960). Eleven randomly selected strains from 10 randomly selected, widely different genera were examined for the presence in the cultures, of cells with different characteristics. On plating these strains under appropriate conditions of media, temperature, and oxygen tension *etc.*, every culture, without exception, gave rise to as great a variety of different colony forms as was found in *Acetobacter* (SHIMWELL, 1958). The correlation of other properties with such mutant colony forms was not specifically studied, but in some cases such correlation forced itself upon the attention, suggesting its presence in others. The genera from which the strains were randomly selected were: *Erwinia*, *Photobacterium*, *Lactobacillus*, *Gluconobacter*, *Serratia*, *Clostridium*, *Zymomonas*, *Flavobacterium*, *Achromobacter*, and *Pseudomonas*. The possibility that a collection of genera again unique in the mutability of its members had been picked seems statistically improbable. If this be accepted, then it seems likely that the majority of bacterial cultures of other genera may show as great a frequency of occurrence of mutants therein as is the case in *Acetobacter*.

Prof. R. E. BUCHANAN (private communication) has remarked that it is strange that the type concept seems to work in other branches of biology but fails in *Acetobacter*. We feel that we may now extend and modify this and say that the type concept seems to apply in the case of the higher organisms, but fails with the bacteria.

If we may now adopt this as a premise, it is suggested that it is the enormously greater relative rapidity of reproduction of bacteria which is responsible for the failure of the type culture concept with them, and its success *e.g.* with higher animals. Prof. D. H. KAY (private verbal communication to one of us — J. G. C.) has expressed surprise that so many bacteriologists should expect bacterial species to remain of constant properties, remarking that after a mammal, for example, had passed through as many generations as does a bacterium in the course of a year or so, he would be surprised if it had remained of the same type.

In the majority of cases each separate "spontaneous" mutation which certain cells in a bacterial culture may undergo usually results, by itself, in only a relatively minor change in that cell's properties, and this minor change may be even less noticeable (by dilution with unchanged cells) in the culture as a whole. However, the cumulative and additive effect of these successive small changes of properties will, in course of time (and often not a very long time, as compared with the higher organisms) result in the characteristics of the culture becoming different from those possessed when the strain was originally isolated, the rapidity and extent of the change depending on the selectivity of the media and the other factors known to influence population changes (*e.g.* BRAUN, 1957).

Eventually, however, what has previously been found to be a relatively stable "major" character may disappear by a sudden loss-mutation, and this may result in a change of species, genus, or even family if the lost character were the chief one on which the taxon was founded. The advent of "quasi-acetobacter" (SHIMWELL and CARR, 1960) is an example of this kind of phenomenon, for if a stable quasi-acetobacter were isolated from some natural habitat there would be no justification for placing it in *Acetobacter*. It would have to be relegated to some vaguely defined genus such as *Achromobacter*.

It has not been our object, in writing this paper, to bolster up a contention that the establishment of *Acetobacter* on the basis of the complete oxidation of ethanol through acetic acid to CO_2 and H_2O is anything more than the only practicable method. We have already pointed out, indeed, (SHIMWELL and CARR, 1960) that "The advent of 'quasi-acetobacters' seems largely to demolish the genus *Acetobacter* whilst failing to indicate an alternative. . . " What we do maintain is that one cannot classify the acetobacters in any other way (ignoring the outstanding character of acetic acid production from ethanol) unless one is content to see them disappear into the depths of some other large vague genus, when even there they would be obviously out of place.

It seems to us that the reason that the type concept succeeds with the higher organisms and fails with bacteria lies in the disparity between two ratios. On the one hand we have the ratio between the generation time of man and that of the bacteria he is studying. Systematic bacteriology is little older than the duration of one man's lifetime, but during that period hundreds of thousands of generations, each involving opportunities for change of characters by mutation or other means, have occurred amongst the bacteria.

On the other hand the ratio of the generation time of man to, say, that of the other higher animals, is small in comparison. Thus in the latter case the type culture appears to remain constant, whilst in the former (the bacteria) the reverse is the case. This being so, we cannot see how any classification of bacteria based on the type species concept (involving the maintenance of type cultures) can be anything more than an ephemeral one. In some relatively stable groups such a classification may appear to remain useful for some years if mass cultures rather than individual cells are examined, whilst in others it will become obsolete in a shorter time. As an actual example, we have found that our own classification of our own collection of *Acetobacter* strains no longer held good after only some six months. Indeed, in some cases strains, upon serial transfer, changed their "species" in a matter of weeks.

Acknowledgements.

We wish to acknowledge our indebtedness to Prof. R. E. BUCHANAN for much stimulating correspondence, without implying that he necessarily agrees with all or any of our views.

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SOME GROWTH EXPERIMENTS WITH *POLYTOMA UVELLA* ON SYNTHETIC MEDIA [1]

by

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INTRODUCTION.

The nutrition of the phytoflagellates belonging to the genus *Polytoma* has been studied by several authors (*e.g.* PRINGSHEIM, 1937; PROVASOLI, 1937; LWOFF and DUSI, 1937).

In growth experiments with *Polytoma uvella*, strain 1, kindly given to us by Prof. PRINGSHEIM, we obtained good growth in a synthetic medium with asparagine as sole source of nitrogen but no development occurred when glycine was substituted for asparagine.

Since such a differential behaviour towards these nitrogen sources had not been reported earlier for *Polytoma* species, the phenomenon was further investigated.

In table 1 the composition of some media used by LWOFF (1932) and by PRINGSHEIM (1937) for *Polytoma obtusum* and *Polytoma uvella sensu stricto* respectively, is compared with that of two media used in the present investigation.

LWOFF and PRINGSHEIM obtained with glycine "good growth" and with asparagine "very good growth". As to asparagine compare ROTTIER, 1936.

When we omitted the yeast extract and the glucose (caramel) from the non-synthetic medium, growth became very poor. Replacing glycine by asparagine always resulted in excellent growth with-

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[1] The contents of this article has been earlier published as part of a doctor's thesis, written in the Dutch language (LINKS, 1955).

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TABLE 1.

Composition of media for *Polytoma uvella* and *Polytoma obtusum* used by various investigators.

Components	Amount in % used by:			
	Lwoff1) (1932)	Pringsheim (1937) 3)	Links (1948)	Links (1952)
Na-acetate	0.1	0.2	0.2	0.2
asparagine or:	0.1	0.1		0.2
glycine	0.1	0.1	0.2	
ammonium salts	0.1	0.1		
glucosamine	0.1	0.1		
cysteine	0.1	0.1		
peptones	0.1	0.1		
K_2HPO_4	0.02	0.01	0.02	0.02
$MgSO_4$	0.01	0.01	0.01	0.01
KCl	0.01			
$CaCl_2$	0.005 2)			
$FeCl_3$	0.005 2)	0.00001		
Na_2CO_3			0.02	0.02
glucose-caramel		0.1	0.2	
yeast extract			10 (v/v)	

All media had the same pH of 7.5 and were made up with distilled water.

1) Compare Lwoff and Dusi (1937 and 1941).

2) Added after sterilization.

3) This non-synthetic medium was based on data of Pringsheim and Mainx (1926) and a personal communication from Pringsheim (1948).

out any added "caramel", yeast extract, $CaCl_2$ or $FeCl_3$. Consequently we decided to investigate the influence of trace elements, thiamin and cyanocobalamin (vitamin B_{12}).

Glycine and asparagine do not serve as carbon-sources (*cf.* Pringsheim, 1937). These substances as well as several yeast constituents, may, however, influence heavy metal ion concentrations by chelate formation (Hutner and Provasoli, 1951).

Vitamins are reported not to be required for *Polytoma uvella* (Provasoli, 1937; Lwoff, 1947; Hall, 1943; Lwoff and Dusi, 1937). But, as the need of thiamin varies strongly within the species of the phytoflagellates (Lwoff, 1947; Lwoff and Dusi, 1937), one could think of a thiamin requiring mutant in the present case. Also it might be conceivable that for a certain phytoflagellate the need for thiamin is greater in a medium with glycine than with asparagine (*cf.* Smyth, 1940). Further, thiamin stimulates carotenogenesis in *Polytoma uvella* (Links *et al.*, 1960).

Yeast extract (Hutner and Provasoli, 1951), asparagine (Robbins *et al.*, 1950) and metabolic products of microbial infections of the cultures (Prings-

HEIM, 1946) can show B_{12} activity and several phytoflagellates are known to need or to be stimulated by extremely small concentrations of this vitamin (LITTLE *et al.*, 1951; HUTNER *et al.*, 1956).

The initial diverging results with the two different N-sources could also have been due to accidental contamination of the medium with cotton plug material which may contain thiamin (SCHOPFER and RYTZ, 1937) and other growth factors (HUTNER *et al.*, 1958).

Experimental.

Cultivation.

Cultures were grown in 10 ml medium, made up with distilled water, in 30 ml culture tubes provided with cotton plugs. The tubes had been cleaned successively with a hot soda solution, diluted HCl, tapwater and distilled water. Sterilization: 120° C. for 20 min. Incubation temperature: 23° ± 1° C. Daylight was excluded. Two successive subcultures were made 7 days after each inoculation with 0.03 ml per 10 ml.

Microbial vitamin B_{12} assay.

Vitamin B_{12} activity was determined with *Lactobacillus leichmannii* A.T.C.C. 313 in the medium of SKEGGS (SKEGGS *et al.*, 1950; AUSSEN, 1954).

	Found (in $m\gamma$ B_{12}/g):
asparagine (Hoffmann-La Roche)	1.35 ± 0.02 (in duplicate)
asparagine (B.D.H.)	2.03 ± 0.03 (in duplicate)
glycine (Brocades, Stheeman and Pharmacia)	0.0
Na-acetate (Hopkins and Williams, Analar)	0.0

Results and conclusions.

Replacing glycine by asparagine in the simple medium (Na-acetate, 3 inorganic salts) always restored good growth. The difference between growth on the two media remained upon:

a) Recrystallization of the asparagine (5 × from H_2O) and of the glycine (6 × from H_2O).

b) Addition of combinations of "caramel", $CaCl_2$ and $FeCl_3$ after sterilization.

c) Addition of 10^{-6}–10^{-3} % $ZnCl_2$; according to polarographic determination, the Hoffmann-La Roche asparagine contained between 10^{-6} and 10^{-4} % $ZnCl_2$ and no acetate or glycine.

d) Addition of the ash constituents of asparagine and yeast extract (dry substance 2%).

e) Addition of the trace element mixture of HUTNER *et al.* (1950) (inconclusive results).

f) Addition of thiamin (1.3 mg/l).

g) Addition of vitamin B_{12}, folic acid, $CoCl_2$ or p-amino-benzoic acid (10^{-5}–2.10^{-3} %).

Some *Polytoma* cultures with the basic acetate-glycine medium, without "caramel", $CaCl_2$ or $FeCl_3$, showed good growth after accidental infection with bacteria.

Other *Polytoma* cultures on the same medium at first showed no sign of growth but started to grow quite well about 2 months after inoculation.

The above mentioned results do not give an indication of any influence of trace elements or of the vitamins studied that might offer a clue to understanding why replacing of glycine by asparagine effects such a remarkable improvement of growth.

Apparently asparagine does not only act as a nitrogen source but may have an additional function, probably by its, usually easy, *in vivo* convertibility into the Krebs cycle intermediate, oxalacetate. Accidental infection with bacteria, yeast extract and long adaptation times of pure *Polytoma* cultures on the glycine-acetate medium probably provide small amounts of Krebs cycle intermediates, required for good growth. These could be succinate or malate.

A parallel with our findings of the usually poor growth of *Polytoma uvella* (strain 1) on the glycine-acetate medium and its excellent growth on the asparagine-acetate medium is found in the growth experiments of HUTNER, BACH and ROSS with another phytoflagellate, the Z strain of *Euglena gracilis*, published, independently and almost simultaneously with ours (HUTNER *et al.*, 1956; LINKS, 1955). These investigators found that their organism utilized its C-substrate (sugar) vigorously if readily available nitrogen and a Krebs cycle intermediate (or auxiliary substrates readily convertible to such an intermediate) were present: these requirements were met by glutamic acid and aspartic acid, or asparagine.

Summary.

1) *Polytoma uvella* (strain 1) grows excellently on the following basic medium: sodium acetate, 0.2%; asparagine, 0.2%; K_2HPO_4,

0.02%; $MgSO_4.7H_2O$, 0.01%; Na_2CO_3, 0.02%; pH = 7.5 (in distilled water). Caramel, $CaCl_2$, $FeCl_3$ or other additional trace metals are not required.

2) Replacement of asparagine by glycine resulted in very poor growth. Addition of thiamin, vitamin B_{12} or trace elements did not improve the glycine medium. It is suggested that *Polytoma uvella* needs small amounts of a Krebs cycle intermediate for good growth.

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THE WINE YEASTS OF THE CAPE

PART V. — STUDIES ON THE OCCURRENCE OF *BRETTANOMYCES INTERMEDIUS* AND *BRETTANOMYCES SCHANDERLII*[1])

by

J. P. VAN DER WALT and AMELIA E. VAN KERKEN

(Received October 16, 1960).

INTRODUCTION.

Brettanomyces species have been shown to be the main cause of yeast hazes in South African table wines examined by us. Moreover, their incidence in these wines appeared to be significantly higher than that reported for other wine-producing localities (VAN DER WALT and VAN KERKEN, 1958).

Although the *Brettanomyces* species associated with vinous products have almost exclusively been isolated from either finished wines or fermenting musts, these yeasts must nevertheless have a wider distribution than has hitherto been recognized. More precise information of this wider distribution is, however, a prerequisite for any effort aimed at the elimination of these yeasts from wines.

The extensive studies of the Italian investigators failed to demonstrate *Brettanomyces* species as normal components of the yeast flora of the primary fermentation (*cf.* VAN DER WALT and VAN KERKEN, 1958). However, DOMERCQ (1956) reported the isolation of *Brettanomyces* from two out of eighty samples of red must from the Gironde, but was unable to detect these yeasts in any of thirty-eight samples of white must from this locality. This indicated that these yeasts could, under certain conditions, occur in the primary fermentation. This would in part account for the presence of these yeasts in finished wines. With the incidence of *Brettanomyces* species being thirty-fold greater in South African wines than in those of the Gironde, it was conceivable that these yeasts could feature quite prominently in the primary fermentation under local conditions. A detailed study of the yeast flora of the primary fermentation had therefore to be undertaken.

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[1]) Part I, II, III and IV: Antonie van Leeuwenhoek **24**, 239, 1958; **25**, 145, 449, 1959; **26**, 292, 1960.

PEYNAUD and DOMERCQ (1956, 1959) by demonstrating the wider distribution of *Brettanomyces* species in the moist mould growth of subterranean cellars made it clear that an investigation of South African wine cellars and wineries would be advisable, even if these are rather drier than on the Continent.

THE RECOVERY OF *Brettanomyces* SPECIES FROM MUSTS AND WINERY EQUIPMENT.

The present study was restricted to the study of white musts because of the prevalence of yeast hazes in this wine type.

In an initial study, eleven samples of must were drawn from eleven wineries in the Stellenbosch-Paarl area during the pressing season in February, 1958. *Circa* 750-ml samples of must were collected directly from the presses or from the sedimentation tanks in sterile 1-l Erlenmeyer flasks before the musts had either been treated with sulphur dioxide or wine yeast starters had been added. The spontaneous flora developing in these samples was studied according to the methods developed by CASTELLI (1954) and by DOMERCQ (1956). Although these fermentations proceeded normally, no cultures of *Brettanomyces* species could be effected from any of the samples. As the procedure adopted has the inherent weakness of only permitting the isolation of the predominant components of the yeast flora, the failure to recover *Brettanomyces* species afforded no proof of their absence.

Initial efforts to locate *Brettanomyces* species in wineries and on winery equipment also failed. Employing the customary media and techniques, yeast isolations were attempted from a number of materials from sources such as lees, scraping from leaking vats and bulk tanks, faulty valves, wine residues and drains. However, only cultures of *Saccharomyces cerevisiae*, *Saccharomyces oviformis*, *Saccharomyces elegans*, *Saccharomyces rosei*, *Saccharomycodes ludwigii*, *Pichia membranaefaciens*, *Candida mycoderma*, *Candida krusei* and *Candida boidinii* could be recovered.

It was therefore quite obvious that in trying to recover the slow developing *Brettanomyces* species from materials heavily infected with other yeasts, the customary isolation techniques were inadequate.

However, Beech and Carr (1955, 1958), in their studies of inhibitory compounds for the separation of yeasts from bacteria, reported that *Brett. bruxellensis* and *Trigonopsis variabilis*, unlike all other yeasts tested by them, showed a marked resistance to actidione and sorbic acid and still developed in the presence of 50 p.p.m. actidione plus 500 p.p.m. sorbic acid.

Consequently, the actidione and sorbic acid resistance of *Brett. intermedius* and *Brett. schanderlii* was tested and confirmed. After some experimentation, procedures were evolved which permitted the recovery of *Brettanomyces* species not only from musts but also from a variety of materials heavily infected with other yeasts. Complete inhibition of all other yeasts was not possible. Their presence did not, however, interfere with the development and isolation of *Brettanomyces* species. Two actidione-sorbic acid-resistant organisms gave trouble initially, *viz. Candida boidinii* and *Oospora lactis*. As these organisms do not utilize disaccharides, the risk of their swamping the development of *Brettanomyces* species could be eliminated by the use of maltose and saccharose as carbohydrate source in the enrichment and other media used.

EXPERIMENTAL.

Media

Medium I.

a	Bacto peptone		5 g
	Bacto yeast extract		3 g
	Malt extract		3 g
	Distilled water		780 ml
	pH		4.8
	Sterilization:	10 lb,	15 min.
b	Saccharose		5 g
	Maltose		5 g
	Distilled water		50 ml
	Sterilization:	10 lb,	15 min.

c Sorbic acid 100 ml stock solution (containing 1.340 g potassium sorbate; filter sterilized).

d Actidione 50 ml stock solution (containing 1,000 p.p.m. actidione; filter sterilized).

e Stock vitamin solution 20 ml (containing 200γ biotin, 40 mg calcium pantothenate, 200 γ folic acid, 200 mg inositol, 40 mg niacin, 20 mg *p*-aminobenzoic acid, 40 mg pyridoxine hydrochloride, 20 mg riboflavin, 100 mg thiamine per litre. Sterilization by filtration).

f	Aureomycin	500 mg
g	Chloromycetin	500 mg

a, b, c, d, e, f and *g* are mixed aseptically and distributed as 25 ml aliquots in 50 ml conical flasks.

Medium II.

a	Bacto agar		20 g
	Bacto peptone		5 g
	Bacto yeast extract		3 g
	Distilled water		750 ml
	Sterilization:	10 lb,	15 min. after distribution in 15 ml aliquots.
b	Saccharose		5 g
	Maltose		5 g
	Distilled water		250 ml
	Sterilization:	10 lb,	15 min.
c	Stock vitamin solution		(I, *e*).

To 15 ml of *a*, 5 ml of *b* and 0.2 ml of *c* are added before solidification of the agar base. The pH is then adjusted to between 3 and 4 and plates are prepared.

Medium III.

a	Bacto yeast extract		4.5 g
	Bacto peptone		7.5 g
	Distilled water		1,000 ml
	pH adjusted to		3.7
	Sterilization:	10 lb,	15 min.
b	Saccharose		6 g
	Maltose		6 g
	Distilled water		100 ml
	Sterilization:	10 lb,	15 min.
c	Stock vitamin solution		(I, *e*)

200 ml of *a*, 100 ml of *b* and 3 ml of *c* are mixed aseptically. The medium is then distributed in Struyk tubes of 10 ml capacity.

Material from Wineries and Winery Equipment.

From five wineries in the Stellenbosch-Paarl area, fifty-three samples of material were collected from places and equipment considered likely to harbour these yeasts.

Samples of material to be investigated were introduced in 25 ml aliquots of Medium I. The flasks were incubated at 28°C. and examined after 2 and 4 weeks. In cases where yeast development had occurred, the enrichments were plated on Medium II. The plates were incubated for at least 2 weeks and regularly examined during this period for the appearance of the typically slow-growing *Brettanomyces* species. Selected colonies were brought into pure culture by plating. Where mould contamination was particularly troublesome, a drop of the enrichment culture (Medium I) was inoculated into Medium III in Struyk tubes and these incubated at 28°C. until fermentation ceased. The sediment was then either re-inoculated into Medium I or plated directly on Medium II. This procedure permitted the separation of fermentative yeasts and non-fermentative moulds. Bacterial development was not completely eliminated by Medium I, but this did not generally interfere and when it occurred it was successfully eliminated by Media II and III.

Industrial Musts.

Ten samples of must were drawn from ten wineries in the Stellenbosch-Paarl area during February, 1960. Samples were collected as before, directly from the crushers or sedimentation tanks before addition of sulphur dioxide or starter yeast cultures.

100 ml aliquots of must were transferred aseptically to 250 ml conical flasks and the following additions made:

Actidione	10 mg
Absolute ethanol	5 ml
Chloromycetin	50 mg
Aureomycin	50 mg
Stock vitamin solution	0.5 ml

The flasks were incubated at 28°C. and plated after 3 and 4 weeks on Medium II.

Control samples of must, artificially infected with *Brett. intermedius* were included in the series as check on the dependability of the procedure.

Grapes.

Ten samples of grapes from the Stellenbosch-Paarl area were collected directly in the vineyards or from transport vehicles and transported aseptically to the laboratory by packing in sterile cotton.

Circa 200 g quantities of grapes were macerated in a sterile container and the pulp transferred aseptically to a sterile 1-l conical flask containing:

Actidione	10 mg
Absolute ethanol	5 ml
Chloromycetin	50 mg
Aureomycin	50 mg
Stock vitamin solution	0.5 ml

The flasks were incubated at 28°C. and the contents plated on Medium II after 3 and 4 weeks.

As check on the dependability of the adopted procedure, one artificially infected pulp was included in the series.

Husks and Pomace.

Ten samples of husks and pomace were collected after pressing from ten wineries.

Circa 1 g of the material was inoculated directly into 25 ml aliquots of Medium I and the flasks incubated at 28°C. The contents were plated after 3 and 4 weeks on Medium II. Control samples artificially infected with *Brett. intermedius* were included.

RESULTS.

Material from Wineries and Winery Equipment.

From thirteen of the fifty-three samples investigated, cultures of *Brettanomyces* species were recovered. The results are given in Table 1. Other yeast species recovered from the samples were: *Saccharomyces cerevisiae, Saccharomyces drosophilarum, Hansenula anomala, Torulopsis bacillaris, Pichia membranaefaciens, Candida mycoderma* and *Candida albicans* (found in one sample only).

Industrial Musts.

From the ten musts examined, one yielded cultures of *Brett. intermedius*. Other yeast species recovered from the musts were: *Saccharomyces veronae, Hanseniaspora valbyensis, Candida mellinii, Candida pelliculosa, Candida krusei,* and *Torulopsis* species.

Grapes.

Although cultures of *Brett. intermedius* were readily recovered from the controls, no other *Brettanomyces* cultures were recovered

TABLE 1.

The Recovery of *Brettanomyces* species from Wineries.

Source of Material Investigated	*Brettanomyces* spp. isolated
Scraping of a wooden tub (1 sample)	*schanderlii*
Material from the breeding ground of *Drosophila* spp. in a drain (1 sample)	*schanderlii*
Material from the breeding ground of *Drosophila* spp. on leaking sherry maturation vats (2 samples from two wineries)	*intermedius* *claussenii*
Material from a drain (5 samples from different localities in one winery)	*schanderlii*
Material from manholes in draining system (2 samples from different localities in one winery)	*intermedius*
Mould growth off a leak in a bulk cement tank (1 sample)	*intermedius* *schanderlii*
Material from a cement floor below a leaking valve in a bulk steel tank (1 sample)	*intermedius*

from any of the samples. Other yeast species recovered were: *Hanseniaspora valbyensis*, *Kloeckera magna*, *Candida mellinii*, *Candida parapsilosis* and *Torulopsis bacillaris*.

Husks and Pomace.

Although cultures of *Brett. intermedius* were recoverable from the control, no cultures of this genus could be effected from any of the samples. Other yeast species recovered were: *Saccharomyces cerevisiae* and *Saccharomyces oviformis*.

Discussion.

The recovery of *Brettanomyces* species from a variety of sources within the winery presents conclusive evidence that these yeasts are more widely distributed in the industry than has generally been recognised. Furthermore, these species appear to be not uncommon contaminants wherever wine or wine residues, and probably alcohol vapour, are allowed to accumulate undisturbed in the presence of air. The single isolation of *Brett. claussenii* is of interest in that it is one of the few occasions where a lactose fermenting yeast has been recovered from a vinous source and is the first instance where this species has been isolated from material other than beer.

The recovery of *Brett. intermedius* from only one sample of must indicates that the genus *Brettanomyces* is not a normal and regular component of the yeast flora of the primary fermentation. The recovery of *Brett. intermedius* from a sample drawn directly from the crushers indicates that these yeasts may nevertheless be present at a very early stage of the vinous fermentation.

The apparent absence of *Brettanomyces* species on any of the ten samples of grapes indicates likewise that these yeasts do not form an integral part of the yeast flora of the mature grape. This confirms the results of DU PLESSIS (1959) on studies of the occurrence of yeasts during the development and maturation of the grape berry.

Similarly, the failure to recover *Brettanomyces* species from husks and pomace supports the view that these yeasts do not form a normal component of the flora associated with the sound vinous fermentation.

Consequently, any occasional recovery of *Brettanomyces* species from fresh industrial musts would appear to be due to the sporadic spreading of contamination from the latent sources of infection within the winery to the equipment during the pressing season.

Apart from mechanical dissemination by the use of contaminated equipment, it is not unlikely that other vectors, particularly insects, are also involved in the infection of both musts and wines. DELCOURT and GUYEOT as early as 1910 established the importance of yeast in the feeding of *Drosophila*. Subsequently, the extensive studies of PHAFF, MILLER, RECCA, SHIFRINE and MRAK (1956), CARSON, KNAPP and PHAFF (1956), SHEHATA and MRAK (1952), SHEHATA, MRAK and PHAFF (1955) and PHAFF and KNAPP (1955) have indicated the importance of *Drosophila* in yeast ecology. The recovery of *Brettanomyces* species from actual breeding and feeding grounds of these flies as reported in Table 1 indicates the possibility of *Drosophila* disseminating these yeasts. Under laboratory conditions it was found possible to recover *Brett. intermedius* from triturated flies twenty-four hours after feeding on this yeast. As yeasts do not survive the passage through the digestive tract of the flies (SHEHATA and MRAK, 1951), yeast dissemination due to external contamination of the flies seems likely.

Although the present study has afforded proof of the wider distribution of the genus *Brettanomyces*, information of its occurrence in nature in the absence of the intervention by man, is still lacking. As it seems improbable that these yeasts are restricted to the fermentation industries, a wider distribution of these yeasts can be anticipated. In view of their slow growth and their high nutritional demands, their incidence in nature can be expected to be very low. Localities most likely to harbour these yeasts will naturally have to simulate conditions encountered in the fermentation industry, *i.e.* localities or sources rich in ethanol and fermentable sugars and containing adequate levels of thiamine and biotin, or the presence of auxo-autotrophic micro-organisms which could act as vitamin donors.

Summary.

A method was developed for the recovery of *Brettanomyces* species from materials heavily contaminated with other yeasts.

This method was applied in the study of the occurrence of the genus *Brettanomyces* in the vinification process. It was found that these yeasts are not uncommon contaminants in the winery and on its equipment. These yeasts were, however, only recovered in one of ten industrial musts and could not be recovered from husks, pomace, or fresh grapes. This supports the view that the infection of wines and musts by *Brettanomyces* species is due to contamination spreading from latent foci of infection within the winery.

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PRENATAL IMMUNIZATION FOR THE PROTECTION OF INFANTS AGAINST WHOOPING COUGH

by

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(Received October 10, 1960).

Of all the diseases against which we practise immunization, whooping cough has been the one of greatest danger to the lives of infants. In Canada in the 5-year period between 1953—1957 there were 550 deaths due to this disease. Four hundred and sixteen (76%) occurred in infants under one year of age and 134 (24%) within the first three months of life. Immunization, if started within the first two months of life, may save the lives of some but not all of these infants but it may not be as effective as immunization started at a later age since the infant's ability to produce its own gamma globulin (and hence antibody) is limited during the first few months of life. Not only will the response to the pertussis antigen be less than it would normally be if immunization were started later but if a combined preparation is used the response to other antigens will also be less. Immunization at three months of age or later is probably too late since most of the mortality will have occurred before the full effect of immunization can be realized. It is for this reason that a number of investigators have studied the value of prenatal immunization (LICHTY *et al.*, 1938; MISHULOW *et al.*, 1942; COHEN and SCADROW, 1943; KENDRICK *et al.*, 1945; FISCHER, 1947). The present report deals with our preliminary studies on this subject.

PROCEDURE.

The subjects in our study were volunteers taken from pregnant women attending the Royal Victoria Hospital's prenatal clinic held in the Rosemont Health Centre of the City of Montreal's Health Department. The infants of these women were also available for the study since they attended the City's Health Department Well Baby Clinic operated in the same centre. The volunteers were divided into two groups on an alternating basis. The odd numbers were taken as the test group and were given an alum precipitated vaccine containing 15,000 million killed *B. pertussis* organisms and 2.5 mg of alum phosphate per ml. The even numbers were the controls and they received a placebo containing only the alum phosphate. The course of inoculations was two subcutaneous injections of 1.0 ml spaced one month apart and starting at the sixth or seventh month of pregnancy. Blood samples were taken from the mothers immediately prior to the first injection and again at the time of child delivery. A cord sample was also taken at this time.

The infants were given an alum precipitated triple antigen containing 20 Lf diphtheria toxoid, 10 Lf tetanus toxoid, 30,000 million killed *B. pertussis* organisms and 2.5 mg aluminium phosphate. All children received the same course of injections regardless of whether they were in the "test" or "control" groups. Immunization was started between the second and fourth month and consisted of three 0.5 ml subcutaneous injections spaced one month apart. Blood samples of 5 ml were taken immediately before the first injection and one month after the third. In this study we have used the agglutination titre as the measure of immune response. Field trials in both the United States (Miller *et al.*, 1943; Sako, 1947) and the United Kingdom (Cockburn *et al.*, 1951) have shown a good correlation between agglutination titre and immunity when whole bacterial vaccines were used as the immunizing agent.

Results.

(a) Agglutination Titres Prior to Immunization: The agglutination titres of the sera taken from the babies at birth (from either cord or venous blood) and immediately before immunization at two to four months of age are shown in Table 1. Six of the children were found to have no detectable agglutinins at birth and 14 (50%) had a titre of less than 1 : 40. At three months of age, 20 (54%) were found to have no agglutination titre and 31 (90%) had less than 1 : 40.

TABLE 1.

Inherited antibodies (pertussis agglutinins) in infants at birth and 3 months of age from mothers immunized during pregnancy with *B. pertussis* vaccine.

Age	No. of Infants	Agglutination titres *									Average titre *
		0	4	8	16	32	64	128	256	512	
At birth	28	6	1	3	2	2	2	7	4	1	93
3 months	35	20	6	3	1	1	3	–	–	1	22

* Shown as the reciprocal of the serum dilution.

(b) Post-Immunization Titres: The results of serum titrations taken one month after immunization are presented in Table 2. The results of the two groups, one with inherited antibody and the other without, are shown. In general, the children without inherited antibody showed higher titres than those with inherited antibody — the latter group averaging about half the titre of the former. The differences, however, are so low that too much significance should not be attached to them.

TABLE 2.

Pertussis agglutinins in infants one month after immunization.

Subject	No. of Infants	Agglutination titres *								Average titre *
		4	8	64	128	256	512	1024	2048	
Children with inherited antibody	23	1	1	1	8	8	3	1	0	248
Children without inherited antibody	20	2	2	2	2	–	8	3	1	480

* Shown as the reciprocal of the serum dilution.

DISCUSSION.

Any benefit derived by the infant from prenatal immunization would appear to be the result of passive transfer of antibody *via* the placental route. The evidence available to date suggests that the infant does not produce gamma globulin until after birth so that there appears little likelihood of active immunization taking place in utero. For prenatal immunization to be effective, the injection procedures followed should produce enough antibody in the mother that, when passed on to the infant, will protect the infant until satisfactory active immunization can be accomplished. For practical reasons it must consist of as few injections as possible. The immunization procedures used in this study were considered inadequate. Fifty % of the children at birth and 90% of them at the time active immunization was started were found to have titres considered to be too low for protection. For this reason the course for immunization has been changed. We are still giving two injections, but are starting at the fourth and fifth months of pregnancy. This change in procedure is based on the fact that the maximum agglutination titre for *B. pertussis* occurs approximately three months after the last injection. The highest titres will therefore be present at the time of birth. The results of this latter study are not yet available.

If prenatal immunization is to become a routine procedure, it will have to be shown that the antibody passed to the infant does not interfere with subsequent active immunization — either with pertussis vaccine or with the other antigens if combined preparations are used. Barr *et al.* (1953) have shown that there can be interference with the response of animals to one component of a combined prophylactic, *i.e.*, to the tetanus toxoid fraction of a diphtheria-tetanus toxoid combination, if the animal has diphtheria antitoxin which is present as a result of previous active experience, either by immunization or with the disease. The diphtheria toxoid acts as a booster, but the response to the tetanus toxoid is depressed. The same type of interference, however, does not appear to occur when passive diphtheria antitoxin is present. In our studies (Greenberg and Fleming, 1950, 1951), we have found that the presence of inherited diphtheria antitoxin may delay the production of diphtheria antitoxin following active immunization, but this may be overcome by the use of a strong prophylactic. A review of our studies over the past few years (approximately 500 children) indicates that inherited diphtheria antitoxin does not interfere with antibody production by the other antigens. The results of the present study (Table 2) would suggest that the type of interference in production of homologous antibody noted in diphtheria immunization may also occur in pertussis immunization. Here, too, (not shown in text) there was no apparent interference with either the diphtheria or the tetanus antitoxin production. The number in the study was small, however, and further study is required to ascertain whether higher agglutination titres (inherited) will result in any greater interference to its homologous or to heterologous antigens.

Finally, one must question whether there is still a need for prenatal immunization against whooping cough. The availability of an immunizing prophylactic, of proven value, has done little to change the morbidity rates in Canada but there have been sharp drops in the mortality figures. Significant drops occurred in 1946, 1952 and in 1957. An all-time low of 40 deaths were recorded in 1958. This is shown in Figure 1.

It would be nice to believe that this drop in mortality is due to our present immunization practices. There is reason, however, to doubt this. While it is true that the morbidity figures must be taken with some reservation as to accuracy, and that the figures may be incomplete since whooping cough is not a reportable disease in all of the Canadian provinces, they do indicate that the rate of incidence for this disease has not dropped. Also, while figures for whooping cough in immunized are not available, its occurrence in the immunized and sometimes with severe symptoms is not rare. In Canada, we appear to take pride in our use of fluid toxoids and vaccines. A review of our results with fluid and precipitated vaccines (Table 3) indicates that only between 61 to 77% responded to fluid pertussis vaccine, whereas between 94 and 97% responded to the adsorbed preparation. The drop in mortalities therefore must be attributed to one or both of two factors — the improvement in methods for treating whooping cough or the decrease in virulence of the causative organism. The possible need for prenatal immunization will continue to exist as long as the morbidity rates remain high. Its adoption as a general practice, however, will be dependent upon the development of procedures which will

TABLE 3.

Response in infants to pertussis vaccines.

Type	3 Doses of (ml)	Number	Responders *
Fluid	0.5	87	53 (61%)
	1.0	86	66 (77%)
Alum-phosphate	0.5	109	103 (94%)
	1.0	254	246 (97%)

* As measured by agglutinin response.

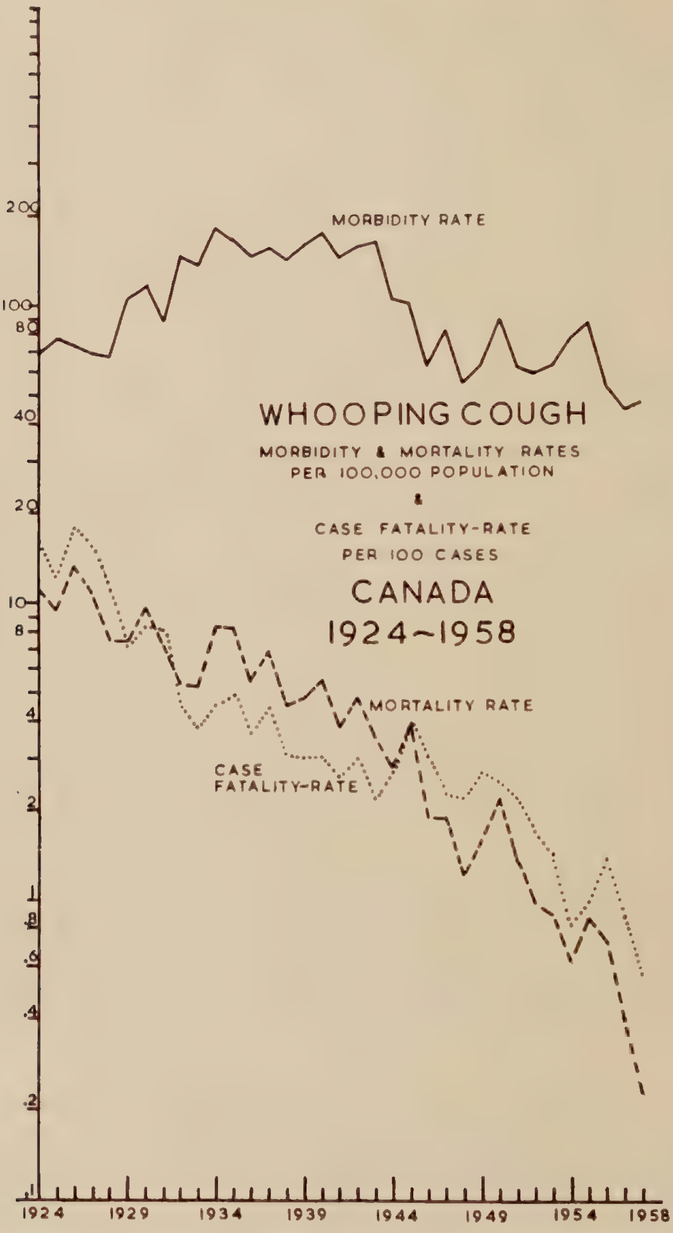
MORBIDITY RATE
WHOOPING COUGH
MORBIDITY & MORTALITY RATES
PER 100,000 POPULATION
&
CASE FATALITY-RATE
PER 100 CASES
CANADA
1924~1958
MORTALITY RATE
CASE
FATALITY-RATE
200
100
80
40
20
10
8
4
2
1
.8
.6
.4
.2
.1
1924
1929
1934
1939
1944
1949
1954
1958

Fig. 1.

yield much better results than those obtained in the present studies. The completion of a large field trial such as has been carried out by the Medical Research Council in the United Kingdom might provide some very useful information.

Summary.

Prenatal immunization against whooping cough could, in theory at least, save the lives of infants. With this in view the present report outlines the results obtained when a group of mothers were immunized with whooping cough vaccine during pregnancy. The immunization procedures followed resulted in low agglutination titres in the mother and subsequently in the infant. As a result, it was necessary to arrange a different schedule of immunizations.

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SEROLOGICAL RELATIONSHIP BETWEEN *TREPONEMA ZUELZERAE* AND THE REITER STRAIN OF *TREPONEMA PALLIDUM*

by

J. H. DE BRUIJN

(Received October 18, 1960).

Introduction.

Recently, Veldkamp (1960) reported the isolation from mud of an anaerobic, free-living spirochete. Difficulties were encountered in classifying this organism since its morphological properties are intermediate between those of the typical *Borrelia* and *Treponema* species. With the cultivable strains of *T. pallidum* it shares the non-pathogenicity but it differs markedly in the nutritional requirements. To study the antigenic structure of the new spirochete, a protein fraction analogous to the Reiter protein antigen (de Bruijn, 1957) was prepared from it and investigated serologically. In these experiments a reactivity with human syphilitic serum was noticed, which lead to further investigations. On account of the results which are reported in this paper, Veldkamp (1960) assigned his spirochete to the genus *Treponema*.

Materials and methods.

Preparation of the protein fraction of *T. zuelzerae*.

The protein fraction was prepared in the same manner as described for the Reiter protein antigen (de Bruijn, 1957) except that ultrasonic disintegration (10 KHz, 250 W) during 5 minutes was substituted for the cryolysis and the salting-out procedure was extended to 100% ammonium sulfate saturation.

Preparation of the lipo-polysaccharide component from the protein fraction of *T. zuelzerae*.

The lipopolysaccharide component was prepared by subjecting the protein fraction to trypsin-digestion according to the method of RAISTRICK and TOPLEY (1934) as previously described in the case of Reiter protein antigen (DE BRUIJN, 1959).

Preparation of a rabbit antiserum against the protein fraction of *T. zuelzerae*.

A rabbit, whose serum was initially non-reactive when tested with the antigens in dilutions equal to those which were used afterwards, was immunized by means of five intravenous injections at three day intervals.

The serum was heated at 61°C. for 30 minutes prior to being tested in the complement fixation test.

Complement fixation test.

The CFT used was a highly standardized routine technique (DE BRUIJN, 1958a). Appropriate serum and antigen controls were included in the test.

Gel diffusion precipitin test.

The slide gel diffusion precipitin test technique of MANSI (1958) was used.

RESULTS AND DISCUSSION.

In preliminary experiments, the protein fraction of *T. zuelzerae* was established to be non-reactive in the CFT with serum of mice which had been infected with *Borrelia duttonii*. When tested in the CFT with human syphilitic serum, it showed a reactivity, similar with that of Reiter protein antigen (table 1). As this reactivity did not diminish when the serum was absorbed with cardiolipin antigen, it cannot be due to a reaction between the reagin of the serum and a cardiolipin-like substance, the presence of which in Reiter protein antigen has been demonstrated (DE BRUIJN, 1958b). Its reactivity with syphilitic serum disappeared, when the protein fraction of *T. zuelzerae* was heated in boiling water for 30 minutes, thus demonstrating the protein nature of the antigenic component.

TABLE 1.

Parallel titration on human syphilitic serum of the protein fraction of *T. zuelzerae* and Reiter protein antigen.

Serum dilutions	Protein fraction of *T. zuelzerae* dilutions						
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
1 : 5	++++	++++	++++	++++	++++	+++	—
1 : 10	++++	++++	++++	++++	++++	+++	—
1 : 20	—	+++	++++	++++	++++	++	—
1 : 40	—	—	—	++++	++++	+	—
1 : 80	—	—	—	—	—	—	—

Serum dilutions	Reiter protein antigen dilutions						
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
1 : 5	++++	++++	++++	++++	++++	++++	++++
1 : 10	++++	++++	++++	++++	++++	++++	++++
1 : 20	++++	+++	++	++++	++++	++++	++++
1 : 40	++	—	—	—	++	++++	++++
1 : 80	—	—	—	—	—	—	+

In a previous paper (de Bruijn, 1959), it was suggested that virulent *T. pallidum* and the avirulent Reiter strain would differ in their polysaccharide fractions. The question rose whether the same difference exists between *T. zuelzerae* and the Reiter treponema.

Analogous to the case of Reiter protein antigen (de Bruijn, 1959), a reactivity was noticed between the homologous rabbit antiserum and the protein fraction of *T. zuelzerae*, heated in boiling water for 30 minutes, which points to the presence of a polysaccharide component in the protein fraction. The unheated protein fraction of *T. zuelzerae* and Reiter protein antigen gave cross reactions with the antiserum of each other, whereas the heated antigens failed to do so, thus suggesting a serological difference between their polysaccharides. To verify this conclusion, the lipo-polysaccharide component of the protein fraction of *T. zuelzerae* was isolated and tested in the slide gel diffusion precipitin test of Mansi (1958) together with the Reiter lipo-polysaccharide. In figure 1 it is demonstrated that both these components are non-reactive with rabbit syphilitic serum and only give precipitates with their homologous antisera.

Absorption of human syphilitic serum with Reiter protein antigen (de Bruijn, 1956), completely removed its reactivity with the protein fraction of *T. zuelzerae*. From this fact and from the

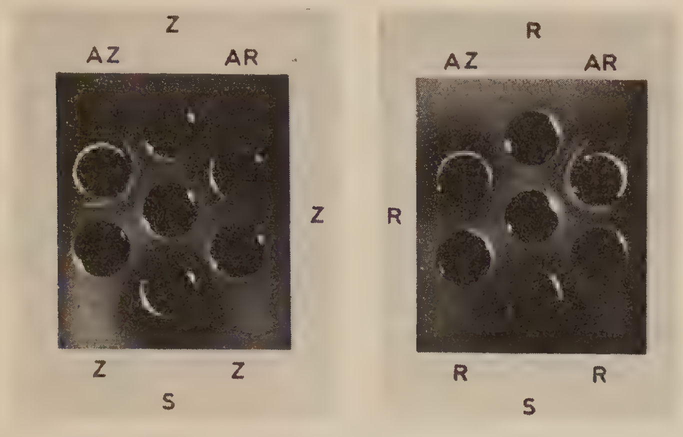

Fig. 1.

Slide gel diffusion precipitin test after 16 hours incubation at 37° C.

Z = *T. zuelzerae* lipo-polysaccharide.
R = Reiter treponema lipo-polysaccharide.
AZ = Rabbit antiserum against the protein fraction of *T. zuelzerae*.
AR = Rabbit antiserum against Reiter protein antigen.
S = Rabbit syphilitic serum (the same results were obtained with human syphilitic serum).

cross reactions of the unheated protein fraction of *T. zuelzerae* and Reiter protein antigen with the antiserum of each other, the presence in *T. zuelzerae* of the 'group specific protein antigen' (Dardanoni and Censuales, 1957; Cannefax and Garson, 1959) is evident.

Summary.

The presence of the 'group specific protein antigen' in *T. zuelzerae* is demonstrated. From the results of complement fixation and gel diffusion tests it can be concluded that this organism and the Reiter strain of *T. pallidum* differ in their polysaccharide fractions.

Acknowledgements.

The author is indebted to Dr H. Veldkamp (Wageningen) for providing a suspension of *T. zuelzerae* and to Dr J. J. Laarman (Leiden) for making available serum of *Borrelia*-infected mice. Thanks are due to Miss J. H. L. de Jong and Mr S. de Bruijn for their technical assistance.

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FREQUENCY OF RESISTANCE TO CHLORAMPHENICOL AND TETRACYCLINES AMONG 12014 *SALMONELLA* STRAINS ISOLATED IN 1958 AND 1959

by

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(Received October 18, 1960).

INTRODUCTION.

It is an established fact that contact with antibiotics may render bacteria resistant and that the frequency of this resistance generally becomes greater the more these substances are used. In 1957 the production of broad-spectrum antibiotics (chloramphenicol and tetracyclines) in the U.S.A. alone amounted to 400.000 kg (DOWLING). When such quantities of extremely potent antibacterial drugs are annually introduced into the biosphere, this is bound to have some effect in the field of microbiology.

One of the chief applications of chloramphenicol is in the treatment of salmonellosis. The tetracyclines are also used for this purpose, but their principal indications lie in other fields. An important application of these drugs is their use in animal feeds. Especially young animals, such as calves, young pigs and chickens usually react to this by an increased growth. Small quantities of tetracyclines given to undernourished infants also have a very favourable effect upon the rate at which they put on weight (MACDOUGAL, 1957).

In most countries the quantities of antibiotics added to animal diets have been considerably increased in the course of the last few years. In the U.S.A. additions of 100 and 200 ppm are by no means exceptional now. Such a development has not taken place in the Netherlands, where the use of antibiotics in cattle fodder has been regulated, the maximum amount allowed being 10 ppm. A serious drawback of the addition of antibiotics to animal feeds is that the intestinal microflora of the animals may become resistant. That this is by no means an imaginary danger, has been shown by the investigations of GRIMBERGEN (1955) and of WILLIAMS SMITH and CRABB (1957). Their observations refer to colibacteria and lactobacilli, bacteria which are generally labelled as non-pathogenic, however.

In the intestinal flora of healthy animals as well as of those suffering from some latent disease *Salmonella* bacteria may be present, and it is especially the resistance of these micro-organisms which may constitute a serious danger (VAN GENDEREN and MANTEN (1955)). HUEY and EDWARDS (1958) also attach great importance to the resistance of *Salmonella*. They did not find a single resistant strain among those isolated before 1948, whereas among the strains dating from the later period (1958) 9% (isolated from birds) and 5% (isolated from human beings) were resistant to tetracycline. The results of their inquiry into the incidence of resistant strains of *S. typhimurium*, partly isolated before and partly after broad-spectrum antibiotics had come into use, may be regarded as indicative of the situation in a country like the U.S.A., where — as stated before — huge quantities of tetracyclines are used in cattle-fodder and as drugs. A multiresistant *S. dublin* was also found in the Netherlands by VAN DER SCHAAF (1957).

The object of the investigation here described was to ascertain what percentage of the numerous strains at our disposal was resistant to chloramphenicol and tetracyclines. In this way we hoped to get some idea of the danger attached to this in our country.

METHODS AND STRAINS USED IN OUR EXPERIMENTS.

The material for this investigation was collected at the National Salmonella Centre, which is a department of the National Institute of Public Health. Here most of the *Salmonella* cultures isolated in the Netherlands are typed. Only strains of human or animal origin isolated in our country have been investigated for antibiotic resistance.

The 12014 strains examined were isolated between May 1958 and January 1960, *viz.*, 9477 from men, 1003 from calves, 413 from other slaughter animals (oxen, horses, pigs, sheep), 185 from other animals (including 24 from laboratory monkeys), 761 from slaughter-houses and surface water, 119 from meat-, bone- and fish meal and

56 from egg products. After serological typing, each strain was inoculated on two agar slopes, one containing 50 μg/ml of chloramphenicol and the other containing 25 μg/ml of tetracycline. These concentrations are roughly 10 times as high as the normal values of the minimum inhibitory concentrations in vitro.

If growth appeared on one or both of the agar tubes after 24—48 hours' incubation, the strain was typed to exclude any possibility of contamination. Only then was the resistance ascertained by

TABLE 1.

Type of insusceptibility of the resistant *Salmonella* strains.

Salmonella types	Number of strains examined	Resistant to			Total number of resistant strains
		tetra-cyclines	chloram-phenicol	tetr. + chlor.	
S. anatum	156	2		1	3
S. bareilly	289	3			3
S. blockley	39	2			2
S. bovis morbificans	273	2		1	3
S. bredeney	773	3	4	1	8
S. california	3	1			1
S. chester	26	1			1
S. dublin	1367	16	1	2	19
S. enteritidis	83			1	1
S. heidelberg	579	6	6	2	14
S. manchester	313	2	1	1	4
S. minnesota	12			1	1
S. muenchen	128	1			1
S. new brunswick	14		1		1
S. newport	486	3	1		4
S. orion	10	1			1
S. panama	13	1			1
S. paratyphi B	446	1	1	13	15
S. saint paul	94	5	7		12
S. schwarzengrund	13	1			1
S. senftenberg	29	1			1
S. stanley	129	1		2	3
S. taksony	59			1	1
S. thompson	38			1	1
S. typhi	91	1			1
S. typhimurium	5767	103	16	25	144
S. worthington	130	2	1	1	3
Other types	654				
	12014	159	38	53	250

means of the usual tube dilution method on slants of 5% sheep's blood agar (pH 7.2—7.4).

RESULTS.

The results of the investigation are given in tables 1 and 2. Table 1 shows that 250 out of 12014 strains that were examined are resistant to tetracycline or to chloramphenicol, or to both. The origins of the resistant *Salmonella* strains are given in table 2.

We found that the chance of finding resistant strains increases in proportion to the number of strains of each type that is examined (table 1). Hence it follows that the ability to develop resistance is not a characteristic of a single serological type or of only a few of these but is, in principle, shared by all Salmonellae. Furthermore, the frequency of resistance depends on the origin of the strains in question (table 2). It is highest in strains isolated from laboratory monkeys. Among strains of human origin and those isolated from calves, resistance is by no means exceptional, either. Among strains isolated from other animals (oxen, horses, pigs, birds, etc.), on the other hand, resistance is comparatively rare. This also applies to strains obtained from slaughter-houses and sewage.

With regard to the type of resistance, we found the following. Out of a total number of 12014 strains 159 were resistant to tetracycline only, 38 to chloramphenicol only, and 53 to both antibiotics; consequently resistance to tetracycline occurred in 212 strains and resistance to chloramphenicol in 91. So the frequency of the former type of resistance is more than twice that of the latter.

DISCUSSION.

As has been stated above, out of a total number of 12014 *Salmonella* strains of various origin examined 250, or 2.08%, proved to be resistant, whereas in the U.S.A. HUEY and EDWARDS (1958) found considerably higher percentages. Compared with the U.S.A., therefore, the situation in our country is rather favourable. A much less favourable picture is presented by the resistance of staphylococci and of tubercle bacteria. In a corresponding period of observation resistance percentages were found for these bacteria of 68% (staphylococci to penicillin) and 22% (tubercle bacteria to isoniazid). That the percentage of resistant *Salmonella* strains is smaller than

TABLE 2.
Origin of the strains

Isolated from:	man	calf	other slaughter animals (ox, horse, pig, sheep)	laboratory monkeys	other animals	slaughter-houses and surface water	meat-bone- and fish-meal	egg products	Total
Number of strains	9477	1003	413	24	161	761	119	56	12014
Number of resistant strains	209	16	3	10	2	4	4	2	250

the figures found for the bacteria just mentioned is probably connected with the fact, observed by some investigators, that *Salmonella* shows little tendency to develop resistance during therapeutic treatment (Russell Twiss *et al.*, 1956; Carnes *et al.*, 1955). However, this is not a universal rule, as appears from the fact that 10 out of 24 laboratory monkeys which had been treated prophylactically and therapeutically with chloramphenicol and tetracyclines were found by us to harbour resistant *Salmonella.* In the case of the 10 insensitive strains isolated from laboratory monkeys there is little doubt as to the cause of this resistance, the animals having been treated with the antibiotics to which the bacteria proved to be resistant. Compared with the small quantities of antibiotics generally used to stimulate growth, the dosage had been relatively high.

As regards the possible cause of the resistance of the 240 other *Salmonella* strains, we may point out the following. In all probability the resistance of the 91 strains that were insensitive to chloramphenicol should also be put down to the use of relatively high doses of this drug. For as a growth-promoting drug — in small doses in cattle-fodder, therefore — chloramphenicol has proved to be of little value, and this more or less excludes the possibility that the resistance may have been due to the fodder the animals had been given. In view of the results of Huey and Edwards, it will hardly be necessary seriously to consider the theoretical possibility that some *Salmonella* strains may have been resistant prior to chloramphenicol being used (natural resistance).

The cause of the resistance to tetracycline seems to be more doubtful, as this drug is used therapeutically in comparatively large doses and in animal feeds in very small ones. Therefore, this type of resistance can theoretically be accounted for in at least two ways.

As the vast majority of tetracycline-resistant strains was isolated from man, resistance in these cases is also likely to have been mainly due to the use of tetracycline in therapeutic doses. In all probability there is a connection between the fact that roughly twice as many of our strains proved resistant to tetracycline as to chloramphenicol and the relatively greater rapidity with which the first type of resistance has generally been found to develop, when these drugs are applied to man. Our opinion as regards the principal cause of resistance to tetracycline is supported by the fact, that many of the resistant strains were not only insensitive to tetracycline, but also to chloramphenicol, which is only used therapeutically.

It is inevitable, that the above discussion should contain an element of speculation, because in most cases there were no data available as regards the nature and the amount of the antibiotics with which the bacteria had been in contact. Moreover, this discussion is based on an investigation which covers only one period. As the use of antibiotics is still on the increase, in medicine and veterinary science as well as in the preparation of animal feeds, we intend to carry out similar investigations in the future. These may enable us to arrive at further conclusions.

Summary.

In the course of 1958 and 1959 12014 *Salmonella* strains were collected and tested for antibiotic susceptibility. 250 or 2.08 per cent of these strains were found resistant to tetracycline, to chloramphenicol or both antibiotics.

In the majority of cases it could be established that resistance was due to the application of these drugs in therapeutic or prophylactic doses.

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(Communicable Disease Center, Public Health Service, U.S. Department of Health, Education and Welfare, Atlanta, Georgia, and National Institute of Public Health, Utrecht, the Netherlands).

SEVEN NEW *ARIZONA* SEROTYPES ISOLATED FROM REPTILES

by

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(Received November 20, 1960).

The cultures to be described all were isolated from the feces of apparently normal captive snakes by the methods ordinarily employed in the isolation of salmonellae. The biochemical characteristics of the cultures were typical of the *Arizona* group.

They were motile, gram negative rods which failed to produce indol, were methyl red positive and Voges-Proskauer negative, grew on Simmons' citrate agar, produced hydrogen sulfide, failed to produce urease or grow in KCN medium, did not utilize D-tartrate, citrate, or mucate when tested by the method of KAUFFMANN and PETERSEN (1956), gave positive tests for the decarboxylation of lysine, arginine, and ornithine, utilized malonate, and liquefied gelatin. Acid and gas were produced promptly from glucose, arabinose, xylose, rhamnose, maltose, trehalose, mannitol, and sorbitol.

Sucrose, raffinose, salicin, inositol, dulcitol, and adonitol were not attacked. Cellobiose either was not fermented or was fermented late and irregularly. Culture 4611-59 did not ferment lactose within 30 days. Culture 598-60 acidified lactose broth after 14 days' incubation. The remaining strains produced acid and gas from lactose within 24 hours.

Culture 4611-59 was isolated from a rattlesnake (*Crotalus terrificus*) [1]. It was agglutinated to the titre of *Arizona* O 1,4 serum and in absorption tests removed all agglutinins from the serum. The

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[1] by P. ZWART, Institute of Tropical and Protozoan Diseases, State University, Utrecht.

H antigens of the culture were monophasic and were agglutinated by H sera for antigens 1,2,5; 1,2,6; 1,6,7; 1,6,7,9; and 1,7,8. When tested with single factor sera for factors 2,5,6,7,8, and 9, agglutination occurred only in factor sera 6 and 7. In absorption tests the organism removed all H agglutinins from 1, 6, 7 serum. The antigenic formula of the organism was 1,4: 1, 6, 7. The O antigens are closely related to *Salmonella* O antigen 53 and the H antigens are related to *Salmonella* H antigen z_4. . . .

Culture 598-60 also was isolated from the feces of a rattlesnake (*Crotalus atrox*) ¹). The O antigens of the culture were identical with those of Arizona O group 1,4 and in agglutination tests with unabsorbed sera the H antigens behaved as did those of culture 4611-59, above. In single factor sera, agglutination occurred in sera 6,7 and 9, and in absorption tests all H agglutinins were removed from 1,6,7,9 serum. The antigenic formula of the culture was 1,4: 1,6,7,9. It is very closely related to the preceding type and its antigenic relations to the *Salmonella* group are the same.

Culture 1393-60 was isolated from a snake (*Bitis gabonicus*) ²). It was agglutinated to the titre of *Arizona* O group 5 serum. In absorption tests it reduced the titre of the serum from 1 : 800 to 1 : 200. Since the culture was not related to other *Arizona* O groups, its O antigen was designated simply as 5, rather than to introduce further complexities into the *Arizona* schema. The H antigens were diphasic. Phase I was agglutinated to the titre of H29 serum, phase 2 to the titre of H25 serum. In absorption tests phases 1 and 2 respectively removed all H agglutinins for the test strains from H29 and H25 sera. The antigenic formula of the culture was 5 : 29 : 25. The O antigen is closely related to *Salmonella* O antigen 48 and the H antigen of phase 1 is related to the *Salmonella* H antigen k.

Culture 96-60 was isolated from a snake (*Bitis gabonicus*) ²). The O antigens were agglutinated to the titre of *Arizona* O group 5 serum but, like those of culture 1393-60, failed to remove all agglutinins from the serum. The H antigens were diphasic and phases 1 and 2 were agglutinated respectively to the titres of *Arizona* H33 and H25 sera and in absorption tests removed all H agglutinins from the sera. The antigenic formula of the organisms was 5 : 33 : 25. The O antigen

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¹) by Dr. J. G. Heyl, Bacteriological and Serological Laboratory, National Institute of Public Health, Utrecht.

²) by P. Zwart, Institute of Tropical and Protozoan Diseases, State University, Utrecht.

is closely related to *Salmonella* O antigen 48 and the H antigen of phase 1 closely related to *Salmonella* H antigen i.

Culture 5716-59 was isolated from an egg eating snake (*Klasypeltis scaber*) [1]. The O antigen of the culture was identical with that of the test strain of *Arizona* O group 24. The H antigens were diphasic and phases 1 and 2 respectively were agglutinated by, and removed all agglutinins from, *Arizona* H sera 23 and 31. The antigenic formula of the organism was 24 : 23 : 31. The H antigen of phase 1 is closely related to *Salmonella* H antigen 1,z_{13} and that of phase 2 to *Salmonella* H antigen z.

Culture 6655-59 was isolated from a tree snake (*Passerita prasina*). The O antigens of the culture were agglutinated to the titre of, and removed all agglutinins from, *Arizona* O group 26 serum.

The H antigens were diphasic and phases 1 and 2 respectively were identical with the H antigens of the test strains of H32 and H30. The antigenic formula of the culture was 26 : 32 : 30. The H antigen of phases 1 and 2 are related respectively to *Salmonella* H antigens c and 1,5.

Culture 5552-59 was isolated from a rattlesnake (*Crotalus terrificus*) [1]. The O antigen of the culture was agglutinated to the titre of *Arizona* O group 26 serum. In absorption tests 5552-59 reduced the titre of the serum for the test strain from 1 : 1000 to 1 : 50. The H antigens were diphasic and the antigen of phase 1 was identical to that of the test strain of *Arizona* H33. The H antigen of phase 2 was identical with that of *Arizona* H31. The antigenic formula of the culture was 26 : 33 : 31. Phase 1 is closely related to *Salmonella* H antigen i, phase 2 to *Salmonella* H antigen z.

Summary.

Seven new *Arizona* serotypes from snakes are described. The biochemical characteristics of the organisms were those of typical members of the *Arizona* group. The antigenic formulae of the types are 1,4: 1,6,7; 1,4: 1,6,7,9; 5: 29: 25; 5: 33 : 25; 24: 23 : 31; 26: 32 : 30; and 26: 33 : 31. Attention is called to the antigenic relationships of the cultures to the *Salmonella* group.

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ACTION DIFFERENTIELLE DE DIVERSES SOUCHES DE *RHIZOBIUM* SUR DES INDICATEURS DE rH[1])

par

P. MANIL et **H. WERNIMONT**

(Reçu le 6 décembre 1960).

Nous avons voulu, au cours des présentes recherches, étudier les points suivants:
a) valeur du test en cause, comme moyen de différenciation, voire d'identification, de souches données de *Rhizobium*;
b) accessoirement, valeur de ce test, en ce qui concerne la différenciation entre souches "effectives" et souches "non effectives", au sein d'un même groupe d'inoculation;
c) valeur de ce test, comme moyen de différenciation de divers groupes d'inoculation.

Pour le premier de ces points il s'agissait simplement de voir, si, dans les conditions de nos essais, l'effet, nul ou plus ou moins accentué, sur des indicateurs ajoutés au milieu, constitue un caractère de netteté et de stabilité suffisantes, en vue de la différenciation de souches de *Rhizobium*.

Le deuxième point présente un intérêt plus particulier. A en juger d'après la littérature, il ne semble exister aucun caractère de laboratoire permettant de prévoir le caractère "effectif" ou non d'une souche donnée.

Or, il fut montré par LEE et WILSON (1943) que l'activité "hydrogénase" est corrélative de l'activité fixatrice, chez *Azotobacter*. Et, en 1957, HOCH, LITTLE et BURRIS détectèrent la présence d'hydrogénase dans les nodules de Légumineuses. N'exagérons cependant pas l'importance de ce fait, car on sait que l'unité fonctionnelle,

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[1]) Recherche subventionnée par l'Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA).

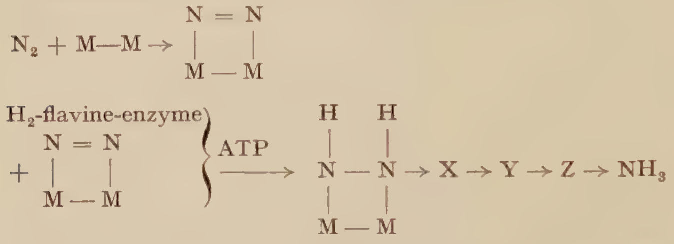
dans la fixation symbiotique, est constituée, non pas par le *Rhizobium* lui-même, mais par la cellule végétale infectée. En culture de laboratoire, le *Rhizobium* ne fixe pas d'azote.

Le schéma de WINFIELD (cité par NICHOLAS, 1958) tente d' expliquer les relations entre hydrogénase et nitrogénase.

a) Hydrogénase:

H_2 + flavine-enzyme $\rightleftarrows$ H_2-flavine-enzyme

b) Nitrogénase:



$$N_2 + M{-}M \rightarrow \begin{array}{c} N = N \\ | \qquad | \\ M - M \end{array}$$

$$\left.\begin{array}{l} H_2\text{-flavine-enzyme} \\ + \begin{array}{c} N = N \\ | \qquad | \\ M - M \end{array} \end{array}\right\} \xrightarrow{ATP} \begin{array}{c} H \quad\; H \\ | \qquad | \\ N - N \\ | \qquad | \\ M - M \end{array} \rightarrow X \rightarrow Y \rightarrow Z \rightarrow NH_3$$

Il s'agit, bien entendu, d'une vue hypothétique où M représente, par exemple, le molybdène.

Par ailleurs, utilisant *Azotobacter vinelandii* et *Clostridium pasteurianum*, NICHOLAS a montré, que Mo exerce son effet sur la fixation de l'azote, alors que l'activité hydrogénase est liée au Fe.

Pour ce qui est du troisième point, au cas où l'action sur les indicateurs de rH ne montrerait qu'une corrélation faible ou nulle avec l'efficience, considérée indépendamment de la spécificité de groupe, il nous a paru intéressant de voir si le caractère plus ou moins réducteur des cultures, dans des conditions données, ne pourrait être lié au "groupe d'inoculation".

Rappelons ici qu'il n'existe aucun caractère certain, morphologique, physiologique ou biochimique, propre à un groupe d'inoculation. Mentionnons au hasard: WILSON, 1939; GREGORY et ALLEN, 1953; BONNIER. 1953; JENSEN, 1958; SMITH, 1958.

Signalons qu'en 1955, sur notre suggestion, une première étude fut effectuée par SEEGER, sur la différentiation des souches de *Rhizobium* au moyen d'indicateurs de rH.

Dans ses essais, SEEGER observait la décoloration plus ou moins accentuée des indicateurs de rH contenus dans le milieu gélosé, ensemencé en surface, en tubes droits. Pour 12 souches de *Rhiz. trifolii* "effectives" utilisées, la méthode montra des différences reproductibles.

Dans le travail de SMITH (1958) nous avons trouvé mention du caractère "réduction du bleu de méthylène", en bouillon et en lait, variable selon les souches de *Rhizobium* observées.

MATÉRIEL ET MÉTHODES.

Souches utilisées. 5 souches de *Rhiz. trifolii* "ineffectives" sur trèfle violet, étiquetées Af 12, Coryn, HKC, Orkney, G 2 et une souche "effective" A 12111, dont provient le mutant ineffectif Af 12. Ces souches nous ont été cédées par P. S. NUTMAN de la Station Expérimentale de Rothamsted, cedont nous le remercions vivement.

12 souches "effectives" de la collection de notre Institut, étiquetées 5.00 - 5.60 - 5.10 - 5.14 - 5.16 - 5.17 - 5.20 - 5.30 - 5.38 - 5.39 - 5.40 - 5.43.

Milieu de culture utilisé. Le milieu de Wright (liquide ou gélosé à 2%), additionné ici d'extrait de levure Difco (1.5 g/litre).

Indicateurs utilisés. Indigotétrasulfonate (E_0 = = —0.03 V à pH 7). Bleu de méthylène ($E_0 = +0.01$ V). Thionine ($E_0 = +0.06$ V).

Au cours d'essais préliminaires, d'autres indicateurs furent essayés, notamment le bleu de toluyle ($E_0 = +0.11$ V) et la safranine ($E_0 = -0.29$ V). Les indications données étant peu nettes, nous avons abandonné ces indicateurs, ne conservant que les trois premiers nommés, sauf dans certains cas.

La technique suivie est mentionnée pour chaque essai. Signalons enfin que chaque essai fut effectué en double et que nous n'avons retenu que les essais donnant régulièrement, pour les 2 tubes représentant un même objet, des indications analogues. Ce qui fut le cas très général.

Essais. Treize essais furent effectués, dans des conditions différentes:

1) Ensemencement, en surface, de tubes gélosés droits, additionnés des indicateurs suivants, à la concentration finale de 2/10.000:

Indigotétrasulfonate;
Bleu de méthylène;
Thionine.

2) Emulsion, dans le milieu gélosé, en tubes droits, avant solidification, de suspensions des divers *Rhizobium*.

Mêmes indicateurs que ci-dessus, mais aux concentrations finales suivantes:

Indigotétrasulfonate : 2/10.000
Bleu de méthylène : 1/10.000
Thionine : 3/10.000.

3) Le milieu gélosé, additionné des 3 indicateurs de rH, fut coulé en boîtes de Pétri. Après solidification, les plaques furent garnies de rondelles de papier filtre (0,8 cm de diamètre) stériles, imbibées d'une culture liquide des divers *Rhizobium.*

4) Addition des indicateurs de rH (mêmes concentrations que pour l'essai 2), à des cultures de 48 h, en milieu liquide, en tubes.

5) Essai semblable à l'essai 2), mais usage des deux indicateurs suivants de E_0 éloignés:

Thionine (E_0 = + 0,06 V)
Safranine (E_0 = — 0,29 V)

6) Essai semblable à l'essai no. 4 (cultures liquides, en tubes), mais addition, à chaque tube, de ½ ml de paraffine liquide, stérile. Celà, afin de limiter l'influence de l'oxygène extérieur.

7) Essai semblable à l'essai no 4, mais portant uniquement sur le bleu de méthylène, et sur des *Rhizobium* isolés de *Trifolium* sp.

8) Addition de l'indicateur de rH (bleu de méthylène), à des émulsions en eau physiologique, de densités optiques égales, de cultures de 24 h des divers *Rhizobium* à l'étude (voir essai no 7).

9) Technique utilisée pour l'essai no 2. Indicateurs: bleu de méthylène et thionine. Examen de souches de *Rhizobium meliloti.*

10) Technique utilisée pour l'essai no 4. Indicateurs: bleu de méthylène et thionine. Examen de souches de *Rhizobium meliloti.*

11) Technique utilisée pour l'essai no 4. Mêmes souches que pour les essais 9 et 10. Indicateurs: bleu de méthylène et thionine.

12) Technique utilisée pour l'essai no 4. Indicateur: thionine. Addition, au milieu, de 2 pour mille de succinate de K.

13) Addition de succinate à des émulsions, en eau physiologique, des *Rhizobium* examinés. Indicateur: thionine.

RÉSULTATS.

Il serait fastidieux de reproduire ici les résultats obtenus pour chacun des 13 essais cités plus haut. Résumons, par exemple, sous forme de tableau, les observations faites au cours de l'essai no 2: émulsion préalable, en milieu gélosé, en tubes droits, de cultures de 24 heures, poussées en milieu liquide (Tableau 1).

Ajoutons que le Tableau 1 n'offre qu'une idée imparfaite des modifications de teinte observée. Seul, des clichées en couleur donneraient

TABLEAU 1.

Décoloration de quelques indicateurs par des souches de *Rhizobium*. Essai no 2.

Indicateurs	Jours	Souches							
		A 12111	Af 12	Coryn	Orkney	G 2	5.00	5.60	1.34 (Luzerne)
Indigo tétrasulfonate	1	—	—	—	—	—	—	+++	+++
	2	—	—	—	—	—	—	+++	++++
	7	++	++	++	++	++	++	+++	+
	9	+++	+++	+++	+++	++	++	+++	—
Bleu de méthylène	1	—	++	+	++	++	+	++	+++
	2	+	++	++	++	++	+	++	+++
	7	+++	+++	+++	+++	++	++	+++	—
	9	++++	++++	++++	++++	++++	++	+++	—
Thionine	1	+	+	++	++	++	++	+++	++++
	2	++	+++	+++	+++	+++	+++	++++	++++
	7	+++	+++	+++	+++	+++	+++	++++	++
	9	++++	++++	++++	++++	++++	++++	++++	+

Remarques: Bleu de méthylène: après 9 jours, recoloration en surface, pour les 5 premières souches (sur 1 cm environ).
Thionine: après 9 jours, recoloration en surface, pour les 6 premières souches (sur $\frac{1}{2}$ à 1 cm environ).
A noter une recoloration totale ou partielle, par la souche 1.34, pour les trois indicateurs.
Signes utilisés: — décoloration nulle; ++++ : décoloration totale.

une image exacte de l'intensité et de l'allure des décolorations notées et de la régularité des résultats, lors des répétitions.

De l'essai no 2 (comme aussi des essais 1, 4 et 5, dont nous ne reproduisons pas les résultats ici), on peut déduire: que, vis à vis des trois indicateurs employés et surtout l'indigotétrasulfonate – des différences marquées peuvent se produire entre les souches, telles les deux souches "effectives" de *Rhiz. trifolii* 5.00 et 5.60. A noter également la recoloration du milieu pour la souche 1.34 (Luzerne).

Dans les essais 1, 4 et 5, utilisant des techniques différentes, les souches 5.60 et 1.34 montrèrent également un pouvoir réducteur accentué dès le début, mais avec, comme pour l'essai 2, recoloration du milieu pour la souche 1.34.

Résumons maintenant, toujours à titre d'exemple, les résultats de l'essai no 7. Cet essai porte sur un ensemble de souches de *Rhiz. trifolii*, toutes "effectives", sauf les souches Af 12 et Coryn, prises ici comme témoins "ineffectifs". Indicateur employé: bleu de méthylène (2/10.000). L'indicateur fut ajouté à des cultures de 24 h, en milieu liquide (Tableau 2).

TABLEAU 2.

Décoloration du bleu de méthylène par *Rhizobium trifolii*, en cultures liquides.

Souches	24 h	48 h	3 jours	5 jours
A 12111	—	—	—	—
Af 12	?	+	++	++
Coryn	—	—	+	++
5.10	?	+	++	++
5.14	—	—	—	—
5.16	—	—	—	—
5.17	?	++	++	++
5.20	—	—	—	—
5.30	+	+	++	++
5.38	—	—	—	—
5.39	?	++	+++	+++
5.40	—	—	—	—
5.43	—	?	++	++
5.60	++	+++	++++	++++

— : décoloration nulle; ++++ : décoloration totale.

Les souches en expérience, toutes spécifiques de *Trifolium*, montrent donc une grande hétérogénéité quant à leur comportement vis-à-vis du bleu de méthylène.

L'essai no 2, exécuté dans d'autres conditions, ainsi que les autres expériences, non relatées ici, fournissent du reste des résultats du même ordre. Bien entendu, si les résultats pour un même essai sont nets, ils diffèrent quelque peu, d'un essai à l'autre, selon la technique employée.

Disons encore que la technique employée pour l'essai no 3 donna des résultats peu intéressants, et que, dans l'essai no 5, la safranine, de E_0 très bas (—0,29 V), ne fut décolorée en aucun cas.

Les essais 9, 10 et 11 portèrent sur 6 souches "effectives" de *Rhiz. meliloti*, mises ainsi en comparaison. Si les résultats furent quelque peu différents, d'un essai à l'autre, ils montrèrent pour un même essai, des différences notables, et parfaitement reproductibles, entre les souches expérimentées.

Les expériences 12 et 13 (présence de succinate) ne fournirent pas d'indications dignes d'être notées.

Les différences observées dans la réduction des colorants ne proviennent du reste pas nécessairement des propriétés biochimiques des souches en cause, mais peuvent être dues également à des propriétés telles que la rapidité du développement dans des conditions déterminées, etc.

Conclusions générales et sommaire.

Nous pensons avoir montré que l'emploi d'indicateurs de rH, ajoutés au milieu, permet de différencier entre elles les souches de *Rhizobium*. Les techniques à utiliser doivent, évidemment, être standardisées. La méthode consistant à émulsionner la préparation bactérienne dans des tubes gélosés additionnés des indicateurs (de E_0 compris entre —0.03 V et 0.06 V), paraît spécialement intéressante.

Il ne paraît pas y avoir de corrélation entre le pouvoir réducteur des souches, et leur "efficience".

En ce qui concerne *Rhiz. trifolii* et *Rhiz. meliloti*, il ne semble y avoir aucune corrélation entre le caractère réducteur et l'appartenance à un groupe déterminé. Toutefois il serait intéressant d'étendre la recherche à d'autres groupes d'inoculation; comme aussi d'étudier l'effet de souches diverses de *Rhizobium*, sur différents substrats, pouvant agir comme ,,donneurs d'hydrogène". Nos recherches se poursuivent dans ce sens.

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SOME OBSERVATIONS ON THE PHYSIOLOGY OF *PSEUDOMONAS NATRIEGENS* NOV. SPEC.[1]

by

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(Received December 10, 1960).

A previously undescribed bacterium isolated from salt marsh mud from Sapelo Island, Georgia, was found to require Na^+ for the induction of enzymes concerned with substrate penetration and utilization (Payne, 1958, 1960). This requirement was not replaceable by a metabolically inert osmotic agent nor by equimolar concentrations of K^+, Li^+, Rb^+ or Cs^+.

This bacterium has the additionally interesting properties of producing enough acid in carbohydrate broth to give a positive methyl red test under the strongly oxidizing conditions of agitated culture and of producing the only bacterial uronic isomerase reported which is specific for glucuronate (McRorie, Williams and Payne, 1959).

The purpose of this paper is to provide additional description of the physiology of this marine bacterium and to give it the specific epithet, *Pseudomonas natriegens*.

Materials and methods.

Morphological and cultural characteristics of the isolate.

The cellular and colonial morphology of this bacterium was described previously (Payne, 1958). Electron micrographs were presented to demonstrate that the cells of the isolate possess polar flagel-

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[1] Supported by research grants G-7128 and G-9865 from the National Science Foundation and by Equipment Loan Contract NR-103-398 with the Office of Naval Research. Contribution no. 26 from the University of Georgia Marine Institute at Sapelo Island.

lation, a property placing the bacterium in the class *Pseudomonadales*. The sensitivity of the organism to 2,4-diamino-6,7-diisopropyl pteridine[1]) (SHEWAN, HODGKISS, and LISTON, 1954) was then determined to indicate whether the organism might correctly be assigned to the genus *Pseudomonas* or the genus *Vibrio*.

Studies of the responses of the bacterium to culture in differential media (PAYNE, 1958) were performed anew after an interval of two and one-half years to reveal any discrepancies in description arising from prolonged culture on laboratory media.

The effect of varying concentrations of sea salt on the growth of the isolate at 30° C. in shaken culture flasks was determined in a medium composed of 2 percent trypticase and varying concentration of sea salt (Sargent) ranging from 0 to 4.5 percent (w/v). Growth was estimated turbidimetrically by means of cuvettes attached to the culture flasks.

The response of the bacteria to varying pH values over the range from 5.5 to 9.5 was determined in a medium containing 2 percent trypticase and 2 percent sea salt. The adjustment of pH was made with NaOH or with H_2SO_4. Cultures were incubated at 30° C. on a rotary shaker and growth was measured as before. Hydrogen ion determinations were made with a Beckman pH meter at zero time and after 8 hrs.

The production of acids in glucose broth and the effect of these metabolic products on growth were observed in cultures of the bacteria in sea water nutrient broth containing 1 percent glucose (w/v) incubated at 30° C. on a rotary shaker.

Products of glucose catabolism.

The products of aerobic dissimilation of glucose were determined in a "fermentation" train like that described by PELCZAR, HANSEN and KONETZKA (1955). In the test system, a mineral salts medium consisting of Na_2HPO_4, 0.3 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.05 g; $(NH_4)_2SO_4$, 0.05 g; $MgSO_4.7H_2O$, 0.005 g; NaCl, 1.5 g and glucose, 1.0 g per 100 ml and adjusted to pH 7.5, was inoculated with 0.1 ml of an 18 hr culture in this same medium and incubated at 25°–30° C. for 72 hrs. Sterile, carbon dioxide-free air was bubbled continuously through the culture.

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[1]) This test was suggested to us by Dr. EINAR LEIFSON, and the reagent was kindly provided by Dr. P. F. D'ARCY, Allen and Hanbury's, Ltd., Ware, Herts., England.

Residual reducing sugar was determined by the method of NELSON (1944). The production of lactic acid was determined by the method described by BARKER and SUMMERSON (1941) and pyruvic acid by the technique described by FRIEDEMANN (1957). Determination of the production of acetic acid was made by a modification of the method of LUDOWIEG and DORFMAN (1960). Carbon dioxide was trapped in a column of ascarite (8–20 mesh) and determined gravimetrically.

Inhibition of induction of oxidative enzymes by chloramphenicol.

HAHN and WISSEMAN (1951) have observed that the induction of enzymes for lactose utilization by *Escherichia coli* is inhibited by chloramphenicol, which is known to inhibit protein synthesis in a variety of bacteria. The sensitivity of the induction of enzymes for the oxidation of glucuronate by this isolate was determined with resting cells harvested from 18 hr agitated cultures in sea water nutrient broth. These cells were washed twice with 0.052 M $MgCl_2$ and suspended in neutralized, aged sea water to give a suspension with 5 percent transmittance at 420 mμ. The substrate was 20μ moles of sodium glucuronate, and 30 μg of chloramphenicol was added to successive respirometer cups at 0, 0.5, 1, 1.5 and 2 hr intervals after the substrate addition. These reaction mixtures were shaken at 30° C., and the rate of oxygen consumption was measured at 15 min. intervals.

RESULTS AND DISCUSSION.

Determinative characteristics of the isolate.

At the beginning of the present study, we had determined that the bacterium should be placed either in the genus *Pseudomonas* or the genus *Vibrio*. A useful and possibly decisive test was available since SHEWAN, HODGKISS and LISTON (1954) observed that *Vibrio* species were distinguishable from non-pigmented *Pseudomonas* species by susceptibility to 2,4-diamino-6,7-diisopropyl pteridine. Our isolate was not inhibited by concentrated solutions of this agent in filter paper discs placed on the surface of sea water nutrient agar plate cultures nor by the powdered substance sprinkled directly on the agar. Therefore, we concluded that the bacterium most probably should be included in the genus *Pseudomonas*.

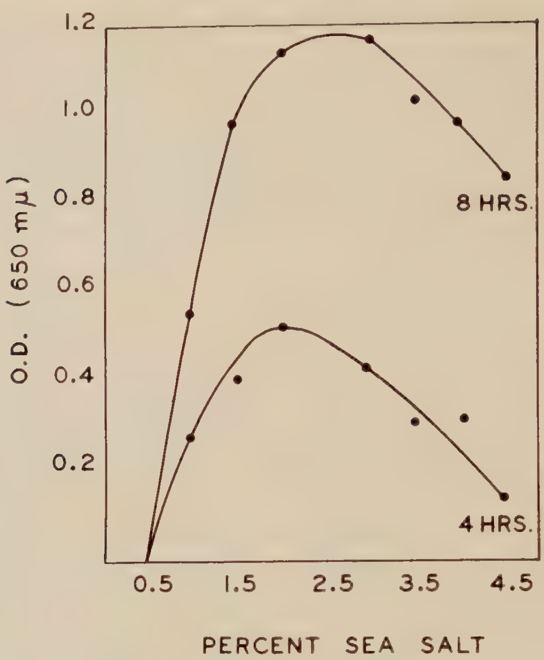

Fig. 1. Effect of sea salt concentration on the growth in agitated cultures of *Pseudomonas natriegens.*

Several significant observations were made from inspection of the responses of the bacterium in differential culture media. Firstly, the bacterium deviated from the previous description in not producing indole and not utilizing inulin, but continued to have the ability to produce acid but no gas with L-arabinose, glucose, fructose, galactose, mannose (±), sucrose, maltose, glycerol and mannitol and not with D-arabinose, xylose, lactose and adonitol. Nitrate reduction, starch hydrolysis and salicin hydrolysis were observed but there was no liquifaction of alginic acid or gelatin, no ureolysis, and the Voges-Proskauer test was negative.

The production of acid in litmus milk occurred slowly, although the bacterium is unable to utilize lactose. Incubation of three or four days was required with the reduction of the litmus occurring two or three days later.

In contrast, we found that the isolate produced acid but not gas in sea water broth containing 0.5 percent sodium glucuronate in only 6-8 hr. No acid was produced with galacturonate even in 14 days. In view of the specificity of the isomerase observed in this isolate for glucuronate (McRorie, Williams and Payne, 1959), these responses to the uronic acids assume considerable importance in the description of the isolate.

The results in figure 1 show that the bacterium does not grow without NaCl and responds optimally to a concentration of 2 to 3 percent in the medium. This property has been chosen as the basis for a specific epithet for the species represented by this isolate. The name *Pseudomonas natriegens* (M. L., *natrium* meaning sodium, and L., *egens*, the participial form of the verb *egere*, to need) is therefore proposed. A culture of the bacterium has been sent to the American Type Culture Collection.

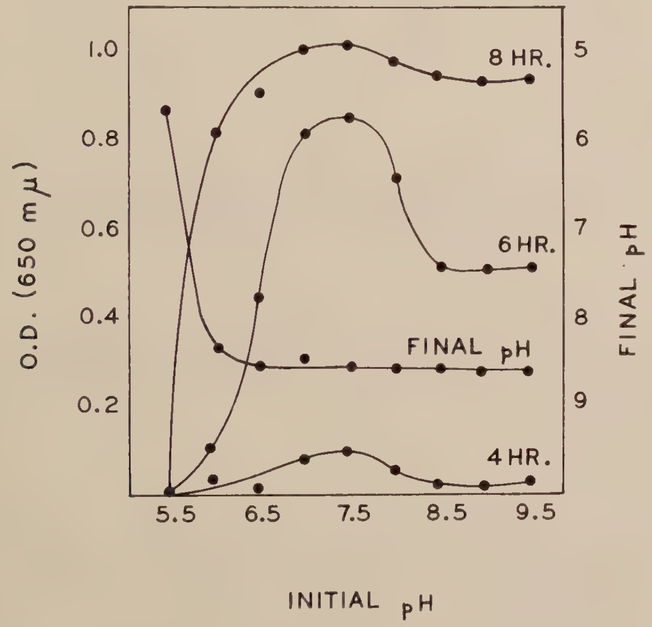

Fig. 2. Effect of initial pH of broth medium on the rate of growth and the final pH of cultures of *Pseudomonas natriegens*.

Additional cultural properties.

The results in figure 2 indicate that growth is optimal if begun at pH 7.5 but can be initiated over the broad range from 6.0 to 9.5. The final pH was quite alkaline in every case. This is characteristic of a number of *Pseudomonas* species owing to ammonia production.

However, the results in figure 3 reveal that growth in trypticase

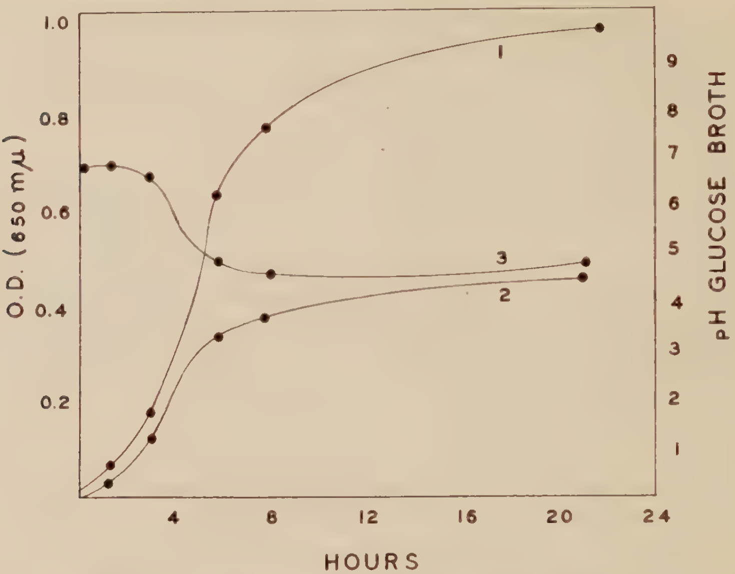

Fig. 3. Inhibition of growth by acid production and pH change in glucose broth cultures of *Pseudomonas natriegens.* 1, growth in sea water nutrient broth; 2, growth in sea water glucose broth; 3, pH of sea water glucose broth cultures.

broth containing glucose is greatly inhibited by the production of acid which sharply lowers the pH of the culture medium. This acid is produced even under the strongly oxidizing conditions of the constantly agitated flask.

Products of glucose catabolism.

The results in table 1 show that approximately 94 percent of the carbon of the glucose metabolized is accounted for in the products

TABLE 1.

Products of glucose dissimilation by *Pseudomonas natriegens.*

mmoles glucose used/ 100 ml	Products	mmoles produced/ 100 ml	% C atoms recovered
2.2	CO_2	2.30	17
	Acetic acid	1.95	30
	Lactic acid	0.85	19
	Pyruvic acid	1.25	28
			94

recovered. These are predominantly acidic and account for the pH changes noted in broth cultures. The enzymology of the dissimilation of glucose by this bacterium is currently being investigated in our laboratory.

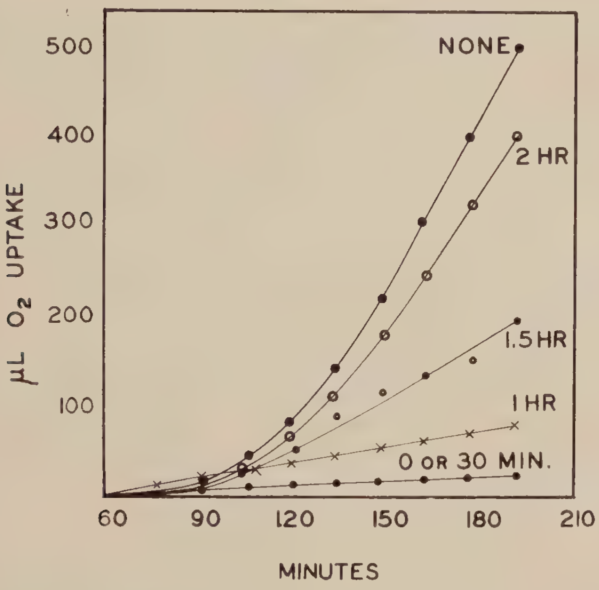

Fig. 4. Inhibition of induction of enzymes for the oxidation of glucuronate by the addition of chloramphenicol to resting cell suspensions of *Pseudomonas natriegens* at various times.

Effect of chloramphenicol on induction of oxidative enzymes.

The results in figure 4 show that the synthesis of induced enzyme protein in resting cells incubated with the inducer glucuronate was inhibited completely by chloramphenicol added at 0 or 0.5 hr. Partial, and proportionally less, inhibition was noted with the addition of the inhibitor during the first 1.5 hr. Synthesis of the induced enzyme appeared more nearly complete after 2 hr, but the activity in the untreated cell suspension was still slightly higher indicating slowly continued synthesis.

The sensitivity of the induction of this system to both Na^+ (PAYNE, 1958; 1960) and chloramphenicol, considered with the fact that induction can be accomplished in resting cells, indicates that the bacterium may be a useful tool for the study of the mechanisms and kinetics of nongratuitous induction of enzymes.

Summary.

Pseudomonas natriegens, nov. spec. which requires Na^+ for growth, has been found to produce a considerable quantity of acid by the dissimilation of glucose in aerobic cultures. The products of glucose catabolism have been identified as CO_2 and acetic, pyruvic and lactic acids. Acid production is very rapid as well in broth cultures containing sodium glucuronate, but not in cultures containing galacturonate.

Growth of this bacterium was not inhibited by saturating quantities of 2,4-diamino-6,7-diisopropyl pteridine. This property indicates that the isolate is more likely to be appropriately placed in the genus *Pseudomonas* than in the genus *Vibrio*.

Induction of enzymes in resting cells for the oxidation of glucuronate was inhibited by chloramphenicol added at various intervals during the first 2 hrs of incubation.

Acknowledgement.

We are grateful for the technical assistance of Mrs. Marcia Wilkins.

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University of Amsterdam).

SOME CHARACTERISTICS OF PATHOGENIC AVIAN PPLO

by

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with the assistance of G. R. van Rhijn and J. de Jong

(Received February 4, 1961).

In a discussion on the nature of the coccoid bodies found in our human PPLO strains (RUYS, 1960; VAN ITERSON and RUYS, 1960*a* and *b*) Dr ROBINOW drew our attention to the description of the so-called Nelson bodies (NELSON, 1936) in cultures of pathogenic avian PPLO. In order to compare these Nelson bodies with our coccoid elements we have studied seven strains of the agent of chronic respiratory disease in chickens (C.R.D.) now called *Mycoplasma gallisepticum* (EDWARD and KANAREK, 1960). Six of these strains were kindly placed at our disposal by Dr M.E.M. STUMPEL, four of which were isolated by himself (TT, K, LB, Blok). One strain originally came from ADLER (S_6) and one from EDWARD (X_{95}). Besides, we also received a X_{95} strain from Dr EDWARD directly.

The strains TT, S_6 and X_{95} were studied extensively. We did not test pathogenicity but all strains agglutinated chicken erythrocytes.

EXPERIMENTS.

Media. We used the following media (STUMPEL, 1959) which are a slight modification of the formula of ADLER (1956):

a. Fluid: peptone-phosphate broth	74	parts, containing:
peptone	20	g
glucose	10	g
NaCl	5	g
$Na_2HPO_4 . 2 H_2O$	2.5	g
phenol red	0.0243	g
aqua dest.	1000	ml
yeast autolysate	16	parts
horse serum	10	parts

b. Solid:	Difco heart infusion agar + 1% glucose	60 parts
	mixture of 16 parts of yeast autolysate 10 parts of horse serum	20 parts

The agar concentration is 1.1%.

Growth. The strains were generally cultivated alternately in the fluid medium (a) and on the solid medium (b). Aerobically and anaerobically growth becomes visible within 24—48 hours at 37°C.

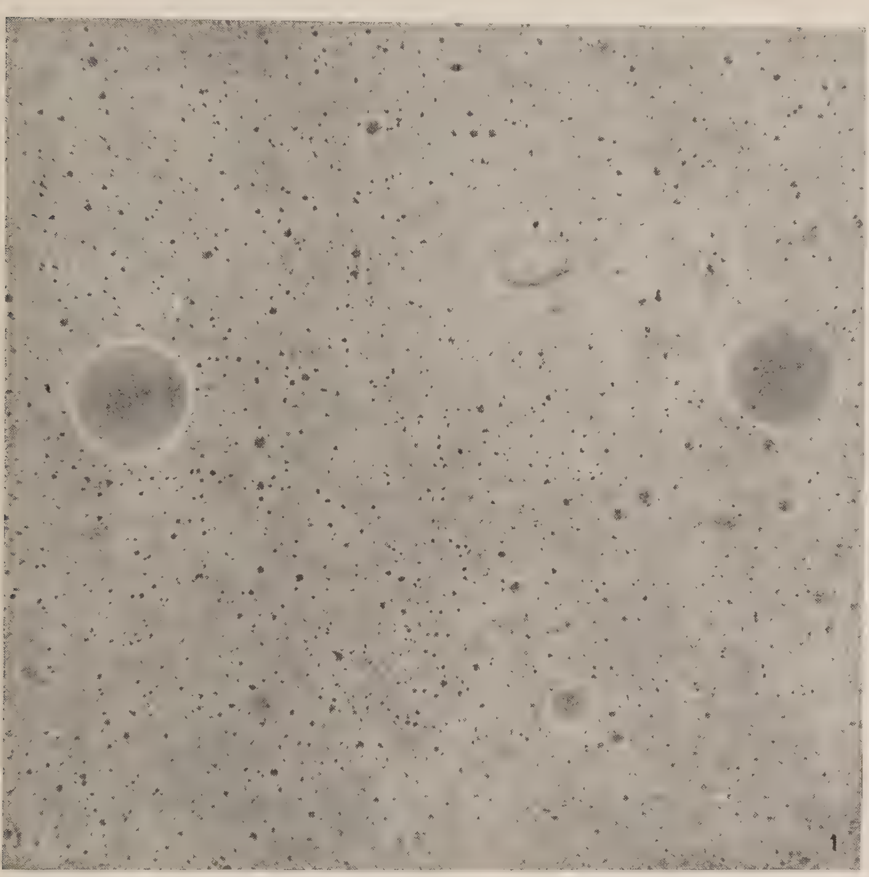
In the fluid medium growth gives only a faint turbidity but it can be deduced from the conversion of the indicator from red to yellow. The cultures are then kept at room temperature and remain viable for another 3 days. In the fluid medium they can be kept in continuous subcultures.

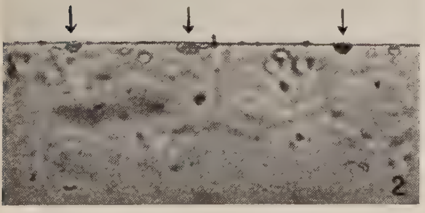
Subcultures from the broth on solid media develop well within 48 hours. When, however, transfers are made from solid medium on to solid medium, either with a loop or by moving a block of agar with the colonies over the surface, growth becomes gradually poorer. It is often lost in the third to sixth transfer.

Colony appearance. In cultures on solid media two types of colonies appear (fig. 1). Generally the majority of the growth consists of very small irregularly formed granular colonies of 15—40 μ in diameter clearly visible with phase-contrast microscopy. Between these small ones large, circular, flat colonies of a regular rather homogeneous, translucent structure appear. Sometimes such large colonies have a darker centre but they never resemble the typical picture of non pathogenic avian PPLO. Their diameter is from 50 to 250 μ. The seven strains studied all showed the same picture, only S_6 grows more abundantly, but there is no essential difference.

Vertical cuts through the colonies (Ruys, 1960) reveal that the small ones penetrate into the medium in the same way as the human and bovine strains do, but they have no brim (fig. 2). The large flat colonies all grow on the surface of the medium only (fig. 3).

In subcultures from solid on to solid medium the large colonies tend to disappear, already in the first transfer. When, however, a block of agar on which only small colonies could be observed was put in the fluid medium the subculture on the solid medium again gave rise to both types of colonies. Generally the number of large colonies became greater the older the broth culture was from which the inoculum was taken.





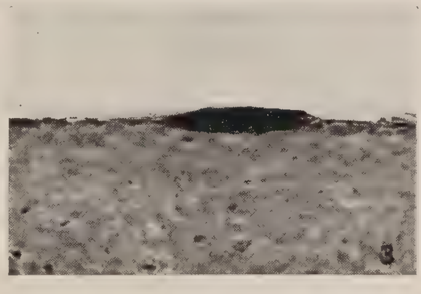


Fig. 1. Agar surface with small and large colonies of TT strain. × 100.

Fig. 2. Vertical cut of small colonies of TT strain. × 270.

Fig. 3. Vertical cut of a large colony of TT strain. × 160.

Attempts to separate the two types of growth. A series of rapid subcultures from fluid into fluid medium did not change the distribution of the types of the colonies which later developed on a solid medium. A whole loopful of material from isolated large colonies inoculated on a solid medium gave rise only to small colonies, which in further subcultures through fluid media again gave both types of development. Separation of the two types of growth by filtration through a membrane filter (pore diameter 450 mμ) was not successful either. From the filtrate only small colonies grew, but later in subcultures the large ones reappeared.

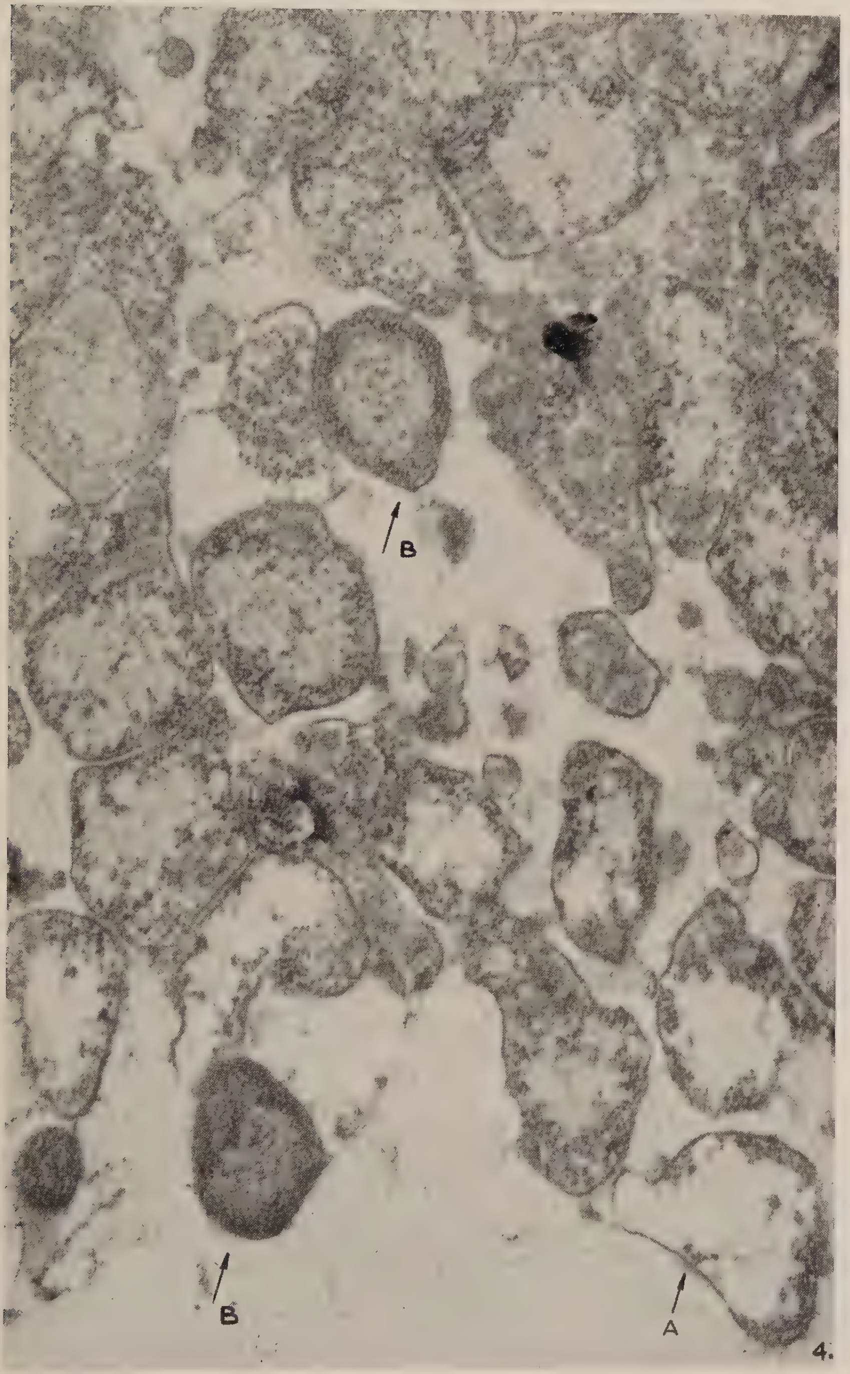
Stained preparations. When preparations from either type of colony were treated with the nigrosin negative staining method only faint ghosts but no sharply delimited bacterial forms could be observed. In Gram stained smears red amorphous masses, threads or granules typical for PPLO appear, but no bacterial forms.

By staining of large colonies on a coverglass with Giemsa solution after Bouin fixation through the agar (Klieneberger's method) small granular structures became visible which resemble Nelson bodies.

The small colonies could not be made visible in the above way with Giemsa because they did not adhere to the coverglass. In order to stain also the elements of the small colonies smears were made of the sediment of cultures in fluid medium. These smears were stained with Giemsa solution after fixation in osmium tetroxide. Their picture then resembled that of human PPLO, showing faintly stained flat vesicles, "amorphous" masses and granules.

Electron microscopy. So far thin sections have been made of the TT strain only. The essentials of the applied technique (after Ryter and Kellenberger (1958)) have been described in

Fig. 4. Electron micrograph of a thin section through a colony of the TT strain of avian PPLO. The edge of a colony is represented after 24 hrs' development in the solid medium with which the inoculum had been thoroughly mixed. At the periphery of a colony the elements appear frequently as irregular vesicles which are poor in internal structure (arrow A). The elements indicated by arrows B in their compactness and inner organization resemble the small coccoid bacteria described for human PPLO (van Iterson and Ruys, 1960 *a* and *b*), but a true bacterial cell wall cannot be seen. Note presence of small elements in the order of size of 100 mμ, whereas the usual order of size is 400 mμ. × 60.000.


B
B
A
4.

our study on human PPLO and their accompanying coccoid bodies (van Iterson and Ruys, 1960 *a* and *b*). In the present investigation the thin sections were made of cultures on solid media embedded in Vestopal W. In some cases we succeeded in sectioning the large colonies separately from the small ones which were more profuse in the embedded material.

With electron microscopy no essential differences could be observed in the elements constituting the two types of colonies. In both colonies the individual units can occur either as irregularly shaped vesicles which are poor in internal structure (arrow A, fig. 4), or as rounded and then usually more compactly filled bodies (arrow B, fig. 4, 5). All transitions between the two extremes seem to occur (fig. 4, 5, 6).

The vesicles are structurally comparable to the elements of human and bovine PPLO in their lack of strict internal organization, and their delimitation by a double contoured membrane.

The compactest elements do resemble in their internal organization (figs. 4 and 5, arrows B) the small coccoid bacteria from our previous study (van Iterson and Ruys, 1960 *a*, fig. 8) in particular in the arrangement of their nuclear material. However, contrary to those cocci a strict cell wall could never be convincingly demonstrated for any of the elements of the TT strain. In the compact elements (arrows B) of the TT strain the organization of the nuclear material shows the general characteristics of bacterial nucleoplasm, *i.e.* a typical delicate fibrillar structure and absence of a nuclear membrane.

Between the vesicles often smaller elements could be discerned

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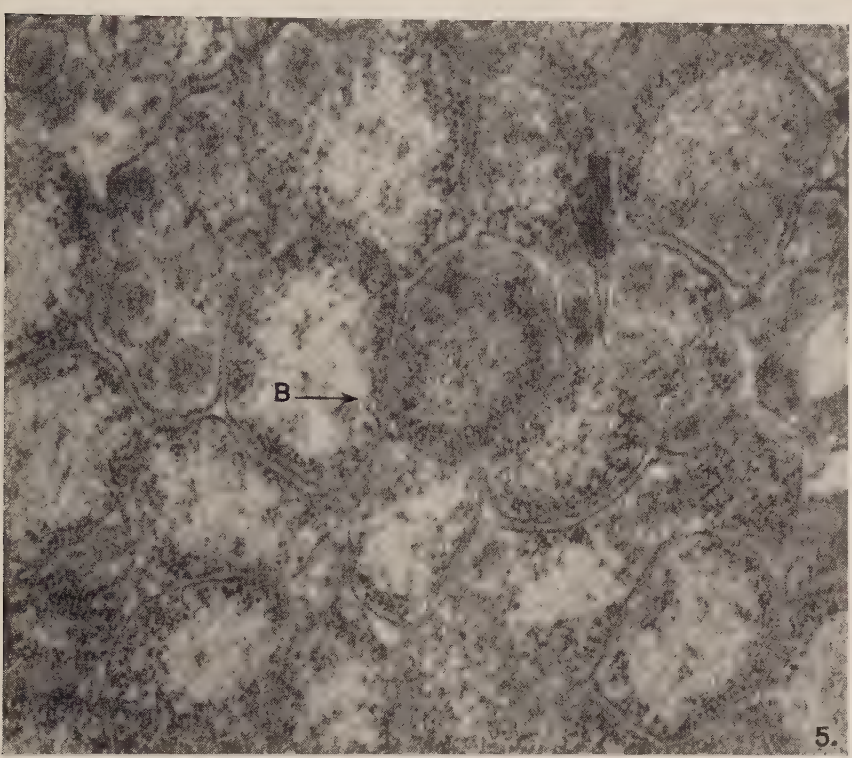
Fig. 5. From a 24 hrs' old colony of the TT strain. At B a body which resembles in its internal organization the coccoid bacteria of human PPLO (van Iterson and Ruys, 1960 *a*), but it misses the true cell wall. The fibrillar organization of its central nucleoplasm appears comparable to that of the nuclear area of bacteria in general. In other elements the nucleoplasms appear in variable grades of dispersion. × 72.000.

Fig. 6. Area from a 24 hrs' old large colony of the TT strain. The elements are frequently rounded in shape. Their delimitation by a double contoured membrane is characteristic and so are the more transparent central spaces containing fibrils of DNA (nucleoplasm) in various degrees of dispersion. These characteristics are shared with other forms of PPLO. × 78.000.



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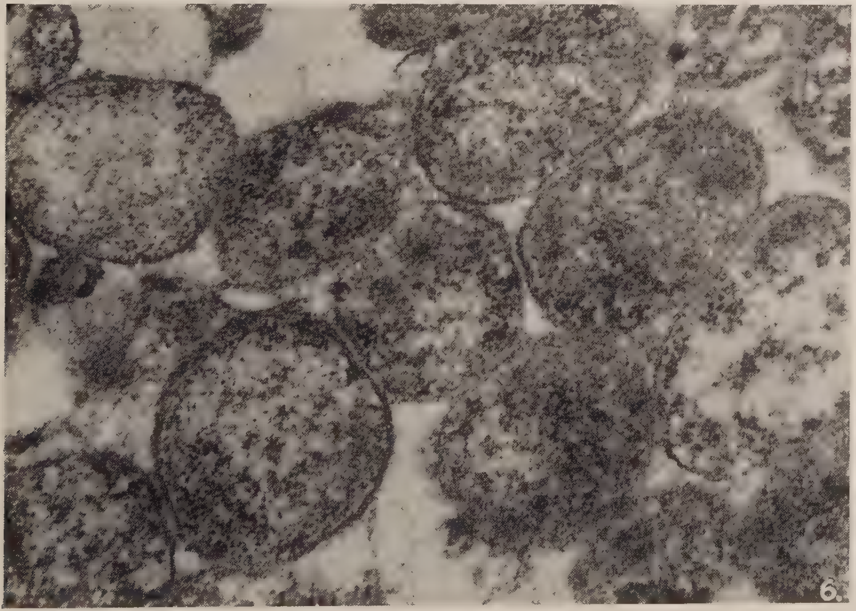

6.

(fig. 4) the significance of which will be discussed in a later communication.

Serology. Antisera were prepared in rabbits by intravenous inoculation of a living culture of the TT, S_6 and X_{95} strains grown in the fluid medium in which the horse serum was replaced by rabbit serum. These cultures contained both types of growth.

Growth inhibition was tested by two methods. Paper disks soaked in undiluted antiserum (heated 30 min. at 56°C.) were put in the middle of the inoculation streak on a solid medium. Another method was to put a drop of serum in the middle of a solid medium and to inoculate the culture over it after it had dried.

Growth inhibition became visible macroscopically with the three sera on all seven strains within 36—48 hours. The sera were not active on a non pathogenic avian strain, nor on the human and bovine PPLO strains tested.

When examined microscopically it became apparent that the small colonies were suppressed, but that the larger colonies had grown, being mostly of smaller size, but with the typical aspect of the large colonies.

For agglutination tests suspensions were made of the large colonies only, because the small colonies are totally embedded in the medium and cannot be scraped off. The tests were made on a slide with undiluted serum. Controls with sera of normal rabbits were negative.

The rabbit immune sera agglutinated the suspensions of the TT strain and other strains.

Three chicken sera which Dr STUMPEL had found to agglutinate his C.R.D. antigen did not cause any growth inhibition of the TT, S_6 or X_{95} strain, but they agglutinated also our suspensions of the TT strain. The serum of a rooster which we had immunized with the sediment of a three times washed culture of the TT strain grown in the fluid medium gave, besides agglutination, some growth inhibition, but much less than the rabbit sera.

DISCUSSION.

In their description of pathogenic avian PPLO strains (*M. gallisepticum*) EDWARD and KANAREK (1960) maintain that the individual colonies may vary in size and are typical for the pleuropneumonia group; ADLER (1960) reports that the colonies never grow larger than 0.5 mm in diameter. Chu (1958) describes that the colonies are generally less than 0.1 mm and usually have no definite centre. No author, however, mentions the existence of two different types of colonies as we have found in all seven strains, the one recently received from Dr Edward included.

A non pathogenic avian strain grew on the same media in only one type of colony which is typical for PPLO. They have a large brim and a circumscript darker centre. In vertical cuts they can be shown to penetrate into the medium.

Our observation with the pathogenic strains that subcultures of the large colonies from solid on to solid medium always give rise to growth of small colonies makes it extremely likely that the small and the large colonies are both growth phases of the same strain. Moreover, with various methods, we did not succeed in separating both types of colonies from each other.

The characteristics of the elements of the small colonies are typical for PPLO. They penetrate into the medium, their growth is suppressed by specific antisera. In the electron micrographs of thin sections the elements are devoid of a true cell wall and in their internal organization they resemble the elements of human and bovine PPLO.

The elements of the large colonies differ from true PPLO in some respects. Their colonies grow on the surface of the media and their growth is inhibited by specific antisera only to a limited extent. The colonies grow on the agar medium impregnated with antiserum but these have a smaller size.

The special character of the elements of the large colonies can also be demonstrated in Giemsa stained preparations made from colonies fixed *in situ* on the medium. The large colonies can be seen to consist of coccoid bodies. The picture of these is conform to that of the so-called Nelson bodies described by Nelson (1936, 1939), Chu (1958) and others. Preparations from the small colonies never show this granular aspect.

With the electron microscope clear-cut differences between elements of both types of growth could not be demonstrated. In both cases the elements are contained in a double contoured membrane, but from our pictures possible differences in the chemical constitution of the thin membranes cannot be deduced. On the other hand, the general characteristics of the Nelson bodies mentioned above seem to point in the direction that their membranous envelopes are of a different quality than those of elements of the small colonies.

In the beginning of our article the question was raised whether the Nelson bodies have the same structure as the coccoid bodies which we have found in our strains of human PPLO. This question must be answered in the negative. The coccoid bodies in the human strains should be considered as true bacteria since they have the typical cell wall and the inner organization of bacteria (VAN ITERSON and ROBINOW, 1961).

However, in the large colonies of the pathogenic avian strains (Nelson bodies) we did find more compactly organized units which in their inner organization resemble the cocci. Such elements might represent transitional stages between PPLO phases and bacteria.

One could suggest a continuous line from PPLO forms, typical Nelson bodies, Nelson bodies with bacterial inner organization to bacterial forms. But we did not yet encounter true bacterial forms in the cultures of pathogenic avian PPLO.

Summary.

Pathogenic avian PPLO, *Mycoplasma gallisepticum* strains were seen to grow on solid media in two types of colonies. The small ones are typical PPLO, the large ones seem to occupy an intermediate position between PPLO and bacteria. The elements constituting the large colonies must be the so-called Nelson bodies.

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"L" FORMS OF *STAPHYLOCOCCUS*

II. STUDIES ON THE MORPHOLOGY OF THE TRANSFORMATION AND ON THE REVERSIBILITY ¹)

by

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with the technical assistance of Mrs. H. A. de Haas-Poppinga, Miss J. van der Veere, Miss J. C. Verhulst and Miss C. H. Blotkamp

(Received January 15, 1961).

In the course of our previous studies on the action of penicillin on staphylococci (1956, 1957) the suggestion was made that a structural change may enable the staphylococci to survive. This supposed change was speculatively associated with the "L" phase. In a later publication (1959) the transformation of the staphylococci into the "L" phase and the reversibility of this phenomenon were described. As "L" colonies were never found on plain agar media containing penicillin, the question was raised whether staphylococci can survive on these media in an intermediary stage between the bacterial and the "L" phase. In order to examine such stages the present investigation was undertaken.

Methods and materials.

Pieces of medium inoculated with staphylococci were cut out and mounted between two coverglasses. The transformation into the "L" phase was studied by phase contrast microscopy. Time-lapse motion picture photographs were made four or eight times every minute. Every single picture of this film was examined in a reading ap-

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¹) Part I: Antonie van Leeuwenhoek **25**, 325, 1959.

²) Working with grants of the foundation "Prof. Dr. D. A. de Jong-Stichting" and the "Stichting ter Bevordering van Medisch-Wetenschappelijk Onderzoek".

paratus and magnified prints were made of many pictures where important stages were considered to be present[1]).

Media.

Except for a minor modification the media used were those described in a previous publication (1959). Dienes' solid medium now used contained:

brain-heart infusion Difco	3.7%
Difco-agar	1.3%
NaCl	3 %
(or KH_2PO_4 5.6%	
or Na_2HPO_4 5.6%)	
$MgSO_4$	0.1%

After sterilisation ascitic fluid to a final concentration of 16,5% was added. Only ascitic fluid of a 30% protein content or more was used. In all penicillin containing media the final concentration was at least 100 U/ml. For the transformation of penicillinase producers into the "L" phase a few additional drops of penicillin 10.000 U/ml and cycloserin (2.5 mg/ml) were added to the medium.

Strains.

All strains in this investigation had been recently isolated from patients. No. 6460, 6988 and 524 were resistant due to penicillinase production, No. 1365 was sensitive to penicillin.

Serology, phage-typing.

We gratefully acknowledge our indebtedness to Dr. OEDING (University of Bergen, Norway) who determined the serological pattern according to a technique published earlier (1957). We are indebted to Dr. R. TH. SCHOLTENS (Rijks Instituut voor de Volksgezondheid, Utrecht), to Professor A. Ch. RUYS and Dr. J. BORST (University of Amsterdam) for phage-typing of the sub-strains reverted from the "L" phase. In all four strains the phage-type of the strains regained was lost after 20 passages in the "L" phase. For this reason the results are not mentioned in the tables. New types were not found.

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[1]) We want to express our gratitude to Prof. P. J. GAILLARD (Leyden University) who gave us the opportunity to use the time-lapse motion-picture apparatus in his laboratory, and to Dr. J. P. SCHERFT for his valuable assistance in making the motion pictures. We are indebted to Mr. J. W. WESSELING and Mr. S. LASZLO for the magnification of these pictures.

Sensitivity pattern.

Sensitivity tests were performed with blotting paper discs; the penicillinase production was determined according to the outline of the sensitivity zone (SCHÖNFELD, 1953).

Survival test.

The number of surviving cocci was estimated by making a replica of the primary penicillin-containing Dienes' media on penicillin-free media, to which commercial penicillinase was added.

Reversibility.

More than 200 subcultures in the "L" phase were made in one year's time. The agarblock technique was used throughout. To regain the coccal phase it was often necessary — especially after the hundredth passage — to change the composition of the penicillin-free media. Sodium chloride was replaced by sodium or potassium phosphate; ascitic fluid was replaced by horse serum (16.5%) or gelatin (5%) and peptone. Media were searched for coccal colonies for at least seven days. Of the many thousands "L" colonies in general only one or a few reverted back to the bacterial phase.

OBSERVATIONS.

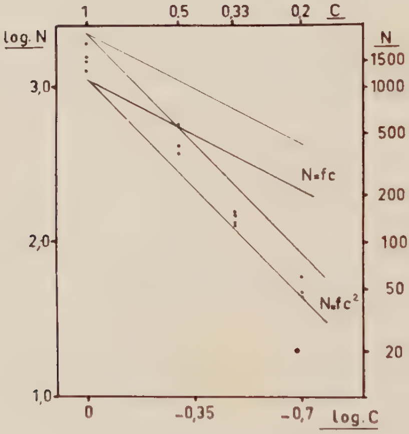
In a preliminary experiment it was found that even on Dienes' medium only a minority of the surviving cocci develop into "L" colonies (table 1). Replica's made at regular intervals on penicillin-free secondary media showed that the *Staphylococcus* colonies on the secondary plates outnumbered the "L" colonies on primary Dienes' medium for at least 72 hours. This means that these bacteria have survived either in the coccal phase or in an intermediary stage.

A chance observation suggested that between the number of cocci in the inoculum and the number of "L" colonies produced no linear correlation exists. If the inoculum was diluted with warm broth 2, 3 and 5 times, the number of "L" colonies decreased approximately 2^2, 3^2 and 5^2 times. The number of original cocci seems to be related to the square root of the "L" colonies produced. This gave rise to the idea that the transformation of cocci into the "L" phase may be conditioned by the association of two factors.

TABLE 1.

Comparison of number of survivors and of number of "L" colonies on Dienes' medium when replica's on media without penicillin are made at different time intervals.

Replica made after hours	Number of "L" colonies on primary plate	Number of staphylococcal colonies on replica plate
24	41	3475
48	40	1231
72	37	259
96	69	51
137	96	41



C	N			
1/1	1520	1770	1300	1490
1/2	560	373	371	417
1/3	131	150	127	155
1/5	27	45	46	60

Fig. 1. Correlation between number of cocci in the inoculum (C = dilution factor of inoculum) and number of "L" colonies gained (N). The lines are drawn for the expected mean values of N plus or minus 2 × the standard-deviation. (For the statistical evaluation we are indebted to Dr. W. Schopman, Rotterdam).

Morphological study.

The pattern of distribution of the cocci remained unchanged on the solid medium used for induction of the "L" forms. Thus the photographs enabled us to trace back the development of the "L" colony up to the very beginning, and to gauge the interval between the different stages (fig. 2—9).

The majority of the cocci seemingly do not change. They are "dormant" and many of them can be revived by adding penicillinase to the medium. Some three or four hours after the inoculation a number of cocci lose their sharp outline, they swell and become flexible vesicles (fig. 6 b). Many of these vesicles appear to burst. We have never observed these vesicles to develop into new elements, either before or after bursting. We repeatedly traced back the first "L" organisms to a dormant coccus (fig. 6 a), but these dormant cocci were usually in the close vicinity of a vesicle. "L" organisms (*i.e.* the first elements that can be distinguished from cocci and that develop into "L" colonies, fig. 3c, 7c) are larger and brighter than cocci. After 6 to 10 hours' incubation they can be distinguished from cocci; the dissimilarity in size, shape and contrast gradually increases (fig. 4 c, 8 c, 9 c). We observed no other stages of development between the coccus and the first "L" organism; the transformation, though completed in a short time, could be followed step by step.

"L" elements develop into colonies by dividing at approximately half an hour's rate. The daughter organisms are connected with fine filaments (fig. 4 c). The network or rootstock thus formed tends to grow under the surface of the agar, it forms the centre of the colony. The periphery is filled with large oval "blebs" (fig. 5d). The first blebs are not formed until a few hours after the initial division of the first "L" elements. Under our experimental conditions these blebs were transparent and optically empty during the first 24 hours; later on their appearance often became spickled, eventually they burst, leaving behind a number of dots. These dots do not seem to have a proliferative function; we were unable to notice any further development. No daughter colonies originated from the periphery of the "L" colonies.

Reversibility.

Another chance observation made us resume our experiments on the reversibility of the "L" phase. Two cultures (originating

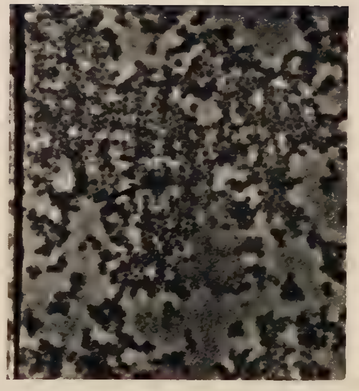


Fig. 2.

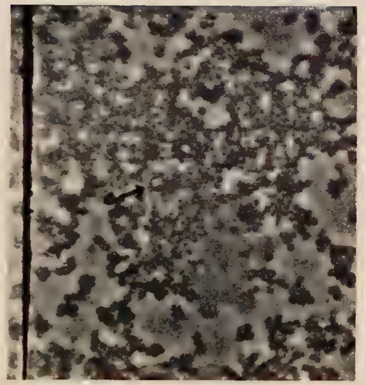


Fig. 3.

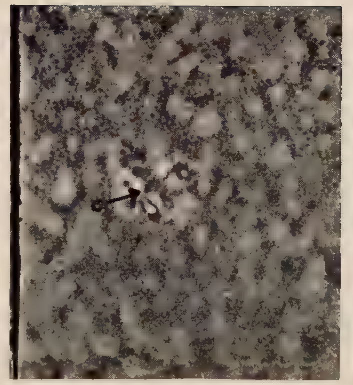


Fig. 4.

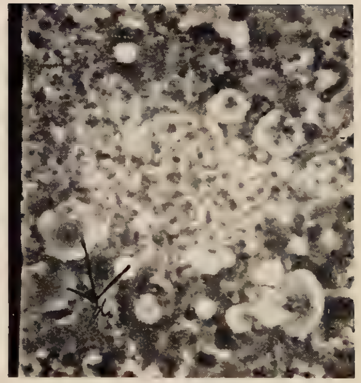


Fig. 5.

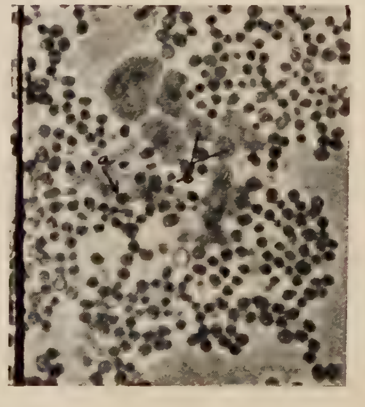


Fig. 6.

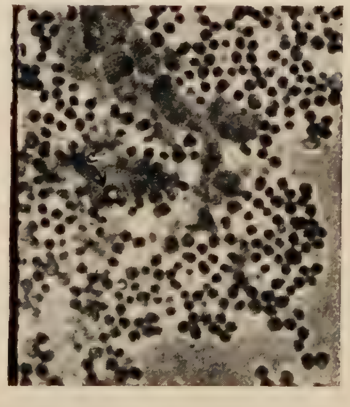


Fig. 7.

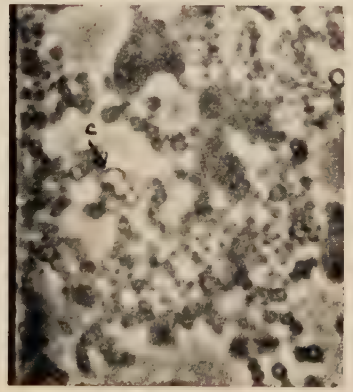


Fig. 8.

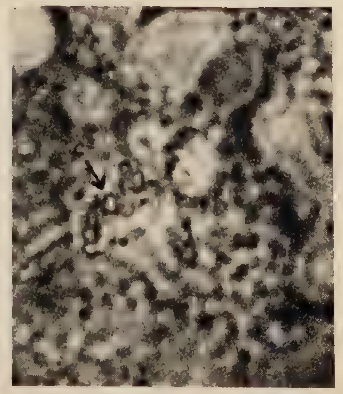


Fig. 9.

Figs. 2-9. *Staphylococcus* strain 5124. Phase contrast microscopy of stages of transformation.

29 X 59	1. after 7½ hours' incubation	magnification 800 ×
	2. after 8 ,, ,,	
	3. after 14 ,, ,,	
	4. after 34 ,, ,,	
17 III 60	5. after 9 hours' incubation	magnification 1300 ×
	6. after 11 ,, ,,	
	7. after 14 ,, ,,	
	8. after 21 ,, ,,	

a) "dormant" coccus; *b*) "vesicle" or swollen coccus; *c*) "L" organism; *d*) "bleb".

from penicillinase producing staphylococci) supposed to be stable in the "L" phase, reverted back to the coccal phase when subcultured from one penicillin-free medium onto another. As far as we know the only difference between the two media was the material of the Petri dishes: the first one was poured into glass, the other into plastic dishes.

Four other strains (three of them penicillinase producers, one sensitive to penicillin) were subcultured in the "L" phase for more than 200 passages. The passages were made on Dienes' medium with penicillin, but subcultures were made on penicillin-free media containing the same concentration of salts and ascitic fluid. We regularly managed to get back a few *Staphylococcus* colonies from many thousands of "L" colonies after a number of intermediate subcultures on media without penicillin. Up to this moment we have never yet succeeded in getting a stable "L" form which could be subcultured indefinitely in the absence of penicillin. Often the "L" colonies reverted back when we changed the medium. *Staphylococcus* 6460 seemed to be "stable" for twenty passages (140—161). When, however, in Dienes' medium the sodium chloride was replaced by potassium phosphate, a few dozen colonies reverted back. Strain 6988, on the other hand, was regained from the 200th passage after only one subculture on a penicillin-free medium.

So far we have failed to define the optimal conditions for the recovery of the *Staphylococcus*, but we have gained the impression that these conditions may be different for various strains. For two of the strains the data for a number of the recovered staphylococci are given in table 2.

TABLE 2.

Properties of staphylococci regained after x passages in the "L" phase.

Strain	Recovered from	Antigenic pattern	Pigmentation	Haemolysis	Coagulase	Mannitol	Penicillinase production (π)
1365	0	ac(h)	+	+	+	+	—
	L_{1}	ac(h)	+	+	+	+	—
	L_{10}	ac(h)	+	+	+	+	—
	L_{23}	a(b)c(h)	+	+	+	+	—
	L_{30}	non typable	+	±	+	+	—
	L_{39}	c(h)	+	+	+	+	—
	L_{50}	(ch)	±	+	+	+	—
	L_{57}	c	±	+	+	+	—
	L_{68}	c	±	+	+	+	π
	L_{78}	c	±	+	+	+	π
	L_{85}	c	±	±	+	+	—
	L_{90}	(c)	±	±	+	—	π
	L_{200}	non typable	—	±	—	—	—
6988	0	ac(e) (i)	+	++	+	+	π
	L_{10}	ac(e) (i)	+	—	+	+	π
	L_{20}	(e) (i)	+	±	+	+	—
	L_{30}	ac(i)	+	±	+	+	π
	L_{40}	(i)	+	±	+	+	π
	L_{50}	ac	+	±	+	+	—
	L_{61}	ac	+	±	+	+	—
	L_{70}	a(b)c	+	±	+	+	—
	L_{80}	ac(e)	+	±	+	+	—
	L_{90}	ac	+	±	+	+	—
	L_{100}	ac	+	±	+	+	—
	L_{200}	(c)	±	—	±	+	π

L_{200} — 200th passage in the "L" phase. 6988 L_{200} — substrain recovered from 200th passage in the "L" phase.

Dr. Oeding (University of Bergen, Norway) kindly investigated the serological pattern of some of the cocci recovered. His comment was: "Gradually reduced serological activity and consequently new antigenic pattern, but no definite new serological type." He endorses our opinion that the substrains are most likely the reverted staphylococci. As in previous investigations only 2—5% of his routine material could not be typed, our untypable substrains 1365 possibly are not contaminants either. Earlier Oeding and Östervold (1959) had found a reasonable stability in the penicillin sensitivity, the phage-type and the serological type of staphylococcus strains which were subcultured in the bacterial phase.

In previous experiments (1959) we found that strains, originally resistant to tetracycline, chloramphenicol and erythromycin may revert to a sensitive mutant after a number of passages in the "L" phase. In this series a chloramphenicol resistant mutant (L_{70}) arose from a sensitive mother-strain 6988. Moreover the results show that penicillinase producers may lose this faculty and that on the other hand penicillin sensitive strains may yield penicillinase producing mutants (table 2).

We managed to retransform into the "L" phase the substrains 6988 regained from every tenth passage up to 200 and of *Staphylococcus* 1365 the substrains 213 and 219. Of the two other staphylococci (524 and 6460) the substrains beyond the 100th passage could so far not be retransformed into the "L" phase.

DISCUSSION.

To our knowledge, all penicillin induced "L" forms of *Staphylococcus* described thus far (DIENES and SHARP, 1956; PROZOROVSKII, 1958, 1959 a and b) originated from sensitive strains. From the present experiments we have gained the impression that any freshly isolated *Staphylococcus* may be transformed into the "L" phase. For the transformation of the penicillinase producers often very high concentrations of penicillin and in addition cycloserin (oxamycin) had to be used (MICHEL and HIJMANS, 1960).

Phase contrast microscopy with time-lapse motion-picture photography gave us the opportunity to study the essential stages in the "L" cycle. In order to avoid confusion descriptive terms were used which do not belong to the standard terminology. Possibly our observations are valid for *Staphylococcus* only and generalisation of our conclusions therefore is not warranted at present.

In our opinion three different groups of organisms are to be distinguished: the "dormant" cocci, the swollen cocci (vesicles) and the "L" organisms. The dormant cocci might be called persistors (BIGGER, 1944); they resume viability when the penicillin in the medium is destroyed. In our earlier publications (1955, 1956) we disputed BIGGER's interpretation according to which persistors are bacteria which by chance do not divide and escape for that reason the action of penicillin. The number of surviving cocci on Dienes' medium appears to be much larger than on plain media; it seems that the number of surviving cocci depends on the medium.

The present experiments contrast in some degree with the interpretation of our earlier experiments done on plain bloodagar media. We assumed that penicillin might cause structural changes, possibly connected with the "L" cycle; we even surmised that desintegration into dwarf forms might have occurred. The present morphological investigation could not confirm the latter supposition. Later experiments may reveal whether the "dormant" cocci, bound to be transformed into an "L" element, lack (part of) the cell wall. In that case these dormant cocci should be considered to be spheroplasts (McQuillen, 1960). At present we are inclined to conclude that in the presence of penicillin bacteria can survive either — for a short time — in the bacterial or — indefinitely — in the "L" phase. We have no evidence that intermediary or side-line stages exist on media containing high concentrations of penicillin.

The swollen coccus or vesicle may assume an enormous size; it probably corresponds with the large bodies described for other species. In our opinion it is part of the bacterial phase; we noticed no further development of isolated swollen cocci either into "L" organisms or back into a coccus. The formation of swollen cocci always preceded the generation of the first "L" organisms; swollen cocci were in the immediate vicinity of the dormant cocci bound to be transformed.

If, as we are inclined to conclude, both dormant cocci and swollen cocci take part in the transformation process this may be a sexual process (conjugation) or a mere cooperation, a growth promotion. The idea that conjugation takes place has been suggested earlier (Klieneberger, 1951; Stempen and Hutchinson, 1951), but to our knowledge only as a preliminary stage to the formation of the large body (our "vesicles"). In our photographic series many vesicles, however, could be traced back to one coccus.

Under the phase contrast microscope only one polymorph type of "L" elements could be distinguished. Dividing cells are brighter, more polygonal than the others, the development is gradual, continuous. Our observations are in agreement with those of Roux (1960) who denies an alternate development of large bodies → granula → large bodies as part of the "internal" "L" cycle. The term large body has given rise to much confusion. We deliberately avoided it so far, speaking about vesicles in the bacterial phase and blebs in the "L" phase. We surmised the function of the vesicle as a factor in the transformation of the dormant cocci into the "L" element and we questioned the function of the "blebs", as has been done earlier by FREUNDT (1958) and LIEBERMEISTER (1960).

Our observations throw some doubt on the existence of penicillin-induced "stable" "L" forms of staphylococci. All our four strains could be reconverted into the coccal form after more than 200 passages in the "L" phase, but usually some of the properties of the original staphylococci were lost after numerous passages; one strain seemed to be stable in the "L" phase for twenty passages without penicillin cover. Later on, however, some colonies reverted back when the sodium chloride in the medium was replaced by potassium phosphate. JOIRIS (1958) described stable phage-induced "L" forms of *Staphylococcus*, but in two of his strains the phage could be demonstrated to be present after respectively 6 and 16 passages. PROZOROVSKII (1959 b) described stable "L" forms of some *Staphylococcus* strains. He subcultured in semi-solid medium. Possibly his inocula were much smaller than ours. This may have reduced the chance of reversion beyond the minimum.

Only recently an "Annotation" in "The Lancet" (1960, II, 250) spoke of "L" forms as of "no more than laboratory artefacts". The only argumentation given was that they never have been found in Nature. We cannot but feel that this statement is rather too positive considering our scanty knowledge of the optimal conditions of growth, of transformation into, and reversion from the "L" phase. DIENES (1960) estimates that: ". . . only a very few organisms, one in many thousands or millions produce progeny". It is not unlikely that our results will be better if our media are less different from the natural habitat — if any — of the "L" forms. Their freakish reversibility should be taken into consideration when discussing their possible rôle in recurrent infections.

Conclusions.

From the morphological study on the transformation of staphylococci into the "L" phase, we are inclined to conclude that intermediary stages between the coccus and the "L" organisms do not exist. Moreover, our results suggest that possibly two different cells take part in the transformation. In spite of two hundred and more passages in the "L" phase, we have not yet succeeded in obtaining stable "L" forms of staphylococci.

Résumé.

Une étude morphologique de la transformation des staphylocoques en phase "L" moyennant un appareil cinématographique nous a suggéré que des stades intermédiaires entre le coccus et les organismes "L" n'existent pas.

Nos résultats nous ont fait considérer la possibilité, que dans la transformation deux cellules bactériennes différentes jouent un rôle.

Malgré que nous avons fait plus de 200 passages en phase "L" nous n'avons pas réussi à obtenir des colonies "L" stables des staphylocoques.

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THE CATABOLISM OF AMINO ACIDS BY ACETIC ACID BACTERIA

by

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INTRODUCTION.

Acetic acid bacteria are well known for their pronounced ability to oxidize a great variety of carbohydrates and related compounds. In this respect they surpass all other bacteria investigated. It would be interesting to know whether the oxidative power of these bacteria also applies to other classes of compounds, the most important being the amino acids. However, although about five hundred publications have been devoted to the physiology and the carbohydrate metabolism of the acetic acid bacteria, only two papers appear to deal with their amino acid metabolism. According to MIYAJI (1924) *Acetobacter rancens* and *A. xylinoides* produce *p*-oxyphenyllactic acid from tyrosine. The latter strain also produces acetic acid from glycine, some succinic acid from glutamic acid, phenyllactic acid from phenylalanine, and imidazollactic acid from histidine. STOKES and LARSEN (1945) investigated the deamination of several amino acids by *Gluconobacter* (*Acetobacter*) *suboxydans*. They found that glutamic and aspartic acids, alanine, lysine, arginine and several other amino acids were deaminated almost to completion after 16 hours of aeration.

This clearly shows that the study of the amino acid metabolism of the acetic acid bacteria has been undeservedly neglected. Accordingly, the current classification of these bacteria and their "species" differentiation is mainly based on their carbohydrate metabolism. It seemed therefore of interest to extend our present knowledge by a more extensive study of their amino acid catabolism.

TAB

Oxidation of several amino acids by acetic acid bacteria. The results are expresse
cases the oxidation still continu

Abbreviations used: n.t. not tested, d. doubtful. The nomenclature of the organisms is

Biotype	Usual "species" designation	Strain number	Biochemical criteria for bacteriological purity
Acetobacter aceti	*peroxydans*	8618	Catalase neg., glucose oxid. neg.
	rancens	23 kl +	Oxid. of glucose to gluconate with uptake of 0.5 O_2/mole substrate.
	lovaniensis	13	idem
	oxydans	8089	idem
	estunensis	E	fructose and gluconate oxid. neg.
	vini acetati	4939	oxid. of glucose with uptake of 1.4 O_2/mole substrate.
	mobilis	6428	erythritol oxid. neg.
	aceti	Ch 31	rapid oxid. of ethanol to acetic acid, followed by slow acetate oxid.
	mesoxydans	4	idem
	mesoxydans	8622	idem
	xylinum	8747	mannose oxid. takes up 0.5 O_2/mole substrate; lactose oxid. neg.
Gluconobacter oxydans	*liquefaciens*	20	rapid oxid. of 2-ketogluconate takes up 0.5 O_2/mole substrate.
	cerinus	21	galactose oxid. takes up 1 O_2/mole substrate; maltose oxid. neg.
	suboxydans	SU,26 117,116	ethanol oxid. takes up 1 O_2/mole substrate; no acetate oxid.
	melanogenus	8086	
	viscosus	8131	
	capsulatus	4939	

METHODS.

Strains used. Several strains of our collection were used, including the most important "species" and representing a fairly general taxonomic survey of the acetic acid bacteria. The origin of these strains, as well as the growth conditions were previously described (DE LEY, 1961). In the present paper (See Table 1) we shall

O_2/ mole substrate after 4 or 5 hours in the Warburg respirometer. In most
nts of the vessels, see text.
as recently proposed in a previous paper (De Ley, 1961).

L-alanine	L-glutamic acid	L-aspartic acid	L-serine	DL-serine	L-threonine	DL-threonine	L-proline
.42	1.81	0.08	0.10	0.23	0.34	0.44	0.96
.93	0.41	1.34	0.08	n.t.	0.57	n.t.	0
.4	0.32	1.2	0	0	0	0	0
.31	0	0	0	0	0	0	0
.12	0.12	0.56	0.06	n.t.	0.04	n.t.	n.t.
.70	0	0.33	0	0	0	0	0.40
.05	0.8	0	0.75	n.t.	n.t.	0.75	0.6
.58	0.91	0.57	n.t.	0.67	n.t.	0.26	n.t.
.18	0	0	1.09	n.t.	0	0	0
.92	0.17	0.09	0	0	n.t.	0	0
.65	0	0.25	0	0	0	0	0.27
.00	0.44	0	0.68	0.60	n.t.	d.	0
.34	0.07	0	0.39	n.t.	n.t.	0.06	n.t.
			no oxygen uptake				

use the nomenclature as recently proposed by one of us (De Ley, 1961).

Preparation of resting cells. After growth, usually for about 2—3 days, the organisms were harvested with a magnetic stirrer from the solid media on Roux flasks, or by centrifugation when liquid media had been used. They were washed three times by suspending them in M/100 phosphate buffer pH 6.0 and centrifuging for about 10 min. at 12,000 g at 4°C. The bacteria were finally suspended in M/50 phosphate buffer pH 6.4 in a concentration of 100 mg of living cells per 1.7 ml of buffer. This suspension was preserved at 0° C. or used at once for the experiments.

Control of bacteriological purity. Acetic acid bacteria are rather slow growing and require a rich medium. It could therefore be expected that occasionally contamination might occur, particularly in liquid media through which a current of air (sterilized over a 10 cm cotton filter) was passed at a rate of 100 L/hour. To check the bacteriological purity of the suspensions of resting cells, we routinely use the following criteria: 1) microscopic aspect ot the cells, (2) the Gram stain and (3) the oxidation of several substrates. The latter point was found to be important, since the former two did not eliminate the possibility of contamination by Gram-negative, small, rod-shaped bacteria. From previous experiments the oxidative behaviour of all our strains was known. For each of them one or more typical reactions were selected (see Table 1) which were known to be characteristic for the strain under investigation. *A. aceti* (*peroxydans*) *e.g.* does not oxidize glucose and lacks catalase; *A. aceti* (*rancens*) oxidizes glucose quantitatively to gluconate; *Gluconobacter oxydans* (*suboxydans*) does not oxidize acetate, etc. Suspensions not conforming to either one of the three criteria were discarded.

Manometric experiments. Oxygen uptake was followed in a conventional Warburg respirometer at 30° C. Each vessel contained 1.7 ml of the suspension (100 mg of living bacteria) in the main compartment, 0.2 ml buffer with 10 μ moles of the amino acid in the side-arm and 0.1 ml 20% KOH in the central well. A control for the endogenous O_2 uptake was always included. It was usually negligibly small. The oxidations were repeated at least three times for every strain. The results were expressed as moles O_2 taken up/mole substrate added. Amino acids of p.a. quality were used in a concentration of 50 μ moles/ml for the L-isomers and 100 μ moles/ml for the DL-mixtures. If necessary the solutions were neutralized to pH 6.4. Insoluble amino acids were used as suspensions.

Amino acid consumption. Disappearance of amino acids was followed by the ninhydrin method according to Moore and Stein (1948). The ammonia liberated was detected with the Nessler reagent.

RESULTS.

1. pH optimum for the oxidation of L-alanine. Preliminary experiments had shown that L-alanine was one of the rare amino acids which were easily oxidized by many strains. Therefore the pH optimum was determined for the oxidation of this substrate. *Gl. oxydans* (*liquefaciens*) was used. The results are presented in Fig. 1 and show that the pH optimum is extended over a wide range from pH 5.8 to 7.1 with the highest value at pH 6.4.

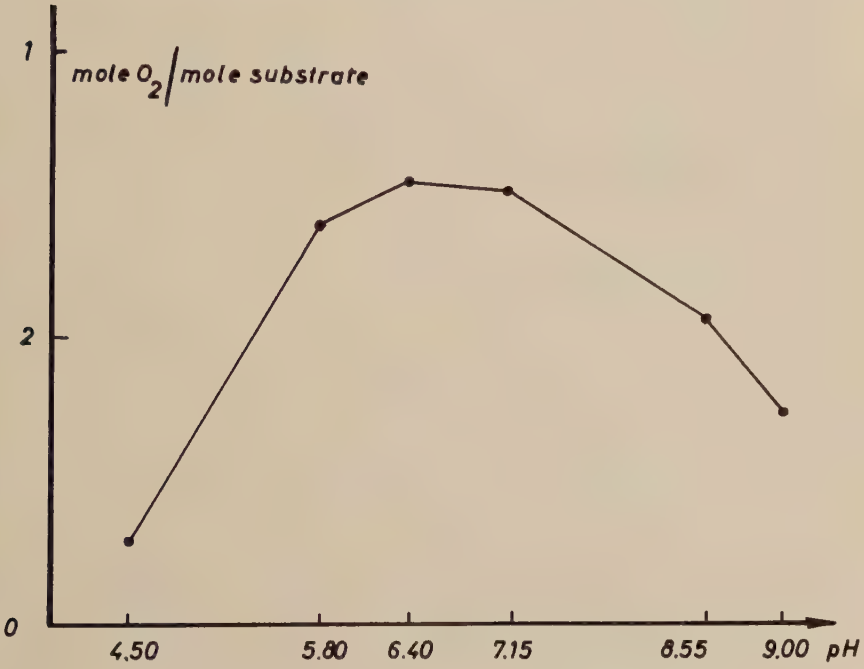

Fig. 1. pH optimum of the oxidation of L-alanine by *Gl. oxydans* (*liquefaciens*). Method: Warburg respirometer. Contents of the vessels: see text.

2. The oxidation of several amino acids. The results are summarized in Table 1 and a few representative oxidation curves are shown in Fig. 2.

2A. The biotype *Acetobacter aceti* and the acetate-oxidizing members of the biotype *Gluconobacter oxydans*. On the whole only three amino acids were more or less easily oxidized: L-alanine, L-glutamate and L-aspartate. The former was by far the most rapidly oxidized. DL- and L-serine, DL- and L-

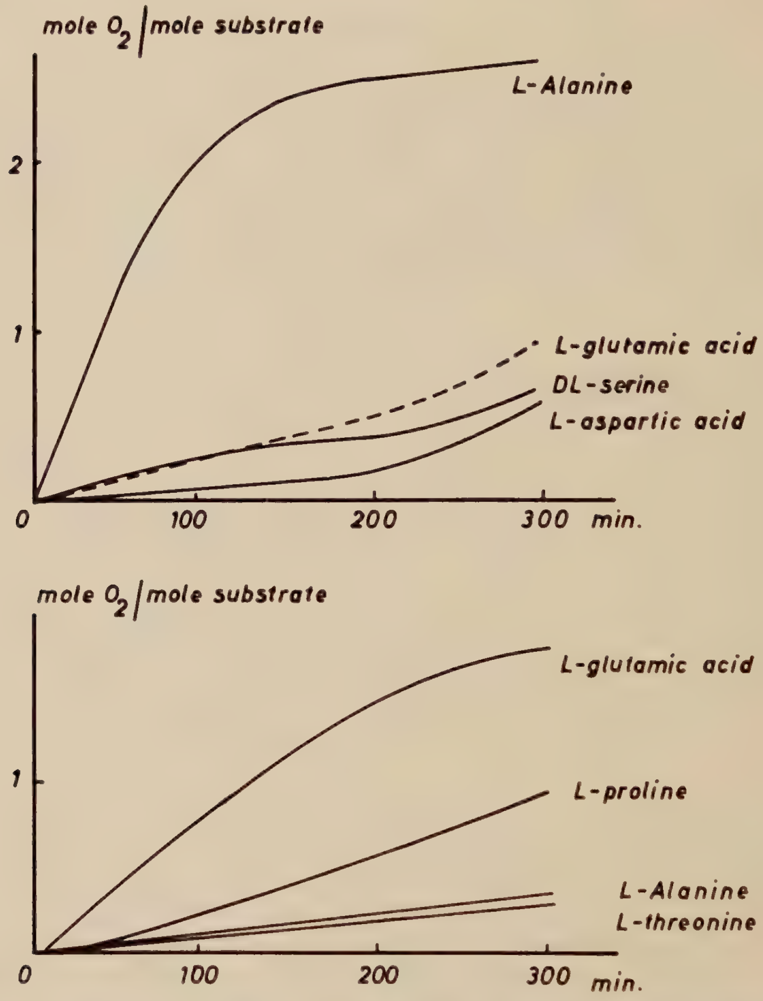

Fig. 2. Oxidation curves of several amino acids by acetic acid bacteria. Method: Warburg respirometer. Contents of the vessels: see text. Upper graph: oxidation by *A. aceti* strain Ch. 31. Lower graph: oxidation by *A. aceti* (*peroxydans*) strain 8618.

threonine and L-proline were usually oxidized slowly and by a few strains only. The following amino acids were not oxidized by any of the bacteria: L-glycine, DL-valine, L- and D-leucine, DL-isoleucine, L-cysteine, L-methionine, DL-phenylalanine, L-tyrosine, L-tryptophane, DL-ornithine, L-lysine, DL-lysine, L-arginine and L-histidine.

2B. The strains of the biotype *Gluconobacter oxydans*, unable to oxidize acetate (the suboxydans group according to FRATEUR (1950)). In this group belong the strains *suboxydans*, *melanogenus*, *capsulatus* and *viscosus*. With none of the above mentioned amino acids could oxygen uptake be observed, in experiments which lasted up to 5 hours. Longer periods were not advisable in view of possible contamination.

3. Assimilation and deamination of amino acids. The disappearance of amino acids was followed in a reaction mixture containing the same concentrations of buffer, bacteria and amino acid as used in the Warburg experiments. The tubes were incubated either at room temperature or at 30° C. and occasionally shaken to prevent precipitation of the cells. In other experiments the suspensions were mixed by a current of air or nitrogen. Samples of 2 ml were taken at definite time intervals, sometimes up to 53 hours; the bacteria were separated by centrifugation for 5 min at 3000 g and an aliquot of the supernatant was used for the ninhydrin reaction. The intensity of the blue colour was determined in the Klett colorimeter with filter 54.

Strains of the *Acetobacter* biotype and of the acetate-oxidizing *Gluconobacter* deaminated the same amino acids which were oxidized in the Warburg apparatus. Amino acids, which were not oxidized, also remained unattacked under the above conditions. *Gl. oxydans* (*liquefaciens*) strain 20 *e.g.* deaminated L-alanine, L-glutamate and serine, but none of the other amino acids tested. The formation of ammonia was confirmed with the Nessler reagent. When nitrogen instead of air was bubbled through the suspension, no deamination was observed which indicated that the latter process was strictly aerobic.

A considerable number of experiments were carried out to detect a deamination process with the *Gluconobacter* strains which did not oxidize acetate. This was not observed under any of the experimental conditions tested. On the contrary, these organisms liberated ninhydrin-positive, Nessler-negative substances very quickly during the first 10-20 min of the incubation period. Negative results were also obtained when mixtures of various amino acids were used, when several vitamins were added or when either glucose or glucose-6-phosphate was added as potential energy source. In some experiments with *Gl. oxydans* (*viscosus*) strain 8131, which lasted up to

53 hours, a deamination of glycine, L-arginine, L-histidine, L-aspartic acid and a few other amino acids was observed, but in these prolonged experiments contamination could not be excluded and they were considered as inconclusive. Since our results were on the whole contradictory to those of Stokes and Larsen (1945), who had used a strain of *Acetobacter suboxydans*, obtained from the University of Wisconsin, we repeated their experiment with our strain SU of *Gl. oxydans* (*suboxydans*). The suspension of bacteria, buffer and amino acid, in the concentrations prescribed by the above authors, was aerated and a trap of N/50 HCl was added for recovery of any NH_3 liberated; however, none could be detected by titration. An additional difficulty was encountered, particularly with the above strain and with *Gl. oxydans* (*viscosus*), which consisted in a spontaneous dissolution of the cells during the aeration process. In a few hours the originally quite turbid suspension had become nearly water-clear. An explanation for this phenomenon can not yet be offered.

Discussion.

In sharp contrast to their pronounced carbohydrate oxidizing capacities, acetic acid bacteria appear to possess only limited abilities to oxidize amino acids. A sharp distinction between two groups was demonstrated: 1) acetate-oxidizing strains (*Acetobacter* biotype and some *Gluconobacter* strains) are able to oxidize and to deaminate a few amino acids; 2) strains, unable to oxidize acetate (most *Gluconobacter* strains, corresponding to the suboxydans group of Frateur) are likewise unable to oxidize and to consume amino acids in periods up to 12 hours. A relationship between amino acid oxidation and the Krebs cycle seems clearly indicated. All the organisms of the former group oxidize alanine, often nearly to completion, since theoretically 3 moles O_2/mole alanine is required and quite often more than 2 moles of O_2 had been consumed. Most of these bacteria also oxidize L-glutamic and L-aspartic acids, but much more slowly. A complete oxidation was rarely obtained, but it seems likely that it was finally achieved, since the reaction progressed continuously during the 5 hour experiment. The oxidative enzymes for serine, threonine and L-proline are very weak and distributed at random through this group.

The inability of resting bacteria from the suboxydans group to oxidize and to consume amino acids is very striking. It poses once more the unanswered question of how these bacteria metabolize their amino acids. The bacteria on a Roux flask are usually full-grown in about three days. The yield is then about 1 gram. One can calculate that this amount of cells contains about 200 mg of amino acids. Assuming an average molecular weight of 180, it follows that 100 mg of living cells are able to assimilate or to synthesize about 18 μ moles of amino acid /12 hours. Our experiments show that the same amount of cells in the same period of time did in fact neither deaminate nor consume any amino acid at all. This tends to show that bacteria from the suboxydans group either synthesize their amino acids *de novo*, or that assimilation (deamination) is correlated with growth and can not be carried out by resting cells at all.

The slow oxidation and consumption of amino acids by many acetic acid bacteria might partially account for the slow growth of these bacteria. *Enterobacteriaceae* and pseudomonads *e.g.*, which are quite often endowed with very active enzyme systems for amino acid metabolism, build up their protein very quickly and quite commonly their cultures are fully grown overnight. The acetic acid bacteria on the other hand, inoculated and cultivated under the same conditions, require several days to one week to reach the same stage.

The inability of strains of the suboxydans group to oxidize amino acids may be used as a quick method to establish whether an unknown strain of acetic acid bacteria belongs to this group. It suffices to wash the cells from a few 2—3 days old slants and to determine the oxygen uptake with ethanol, acetate and L-alanine in the Warburg apparatus. If no oxygen uptake higher than the endogenous one, which is usually quite small, is observed with the latter two substrates, and if ethanol is quantitatively converted into acetate (1 mole O_2/ mole ethanol), the strain belongs to the suboxydans group of *Gluconobacter*. In addition, a flagella stain ought to show polar flagella. This simple procedure eliminates the usual growth experiments which take several days.

Summary.

The catabolism of several amino acids has been studied with resting cells of various strains of acetic acid bacteria. In strong contrast to their pronounced carbohydrate oxidizing capacities, these bacteria possess only limited abilities to catabolize amino acids. All strains from the biotype *Acetobacter aceti* and the acetate-oxidizing

strains of the biotype *Gluconobacter oxydans* oxidize L-alanine often to near-completion. Most of them oxidize L-glutamic and L-aspartic acids, but on the whole much more slowly. Some strains also oxidize serine, threonine and proline weakly. All other amino acids investigated are not attacked. With several strains the deamination was investigated. Only those amino acids are deaminated, which can be oxidized. Resting cells of the biotype *Gluconobacter oxydans* which are unable to oxidize acetate (the suboxydans group according to FRATEUR) are likewise unable to oxidize, to deaminate and to assimilate any amino acid supplied. A simple procedure to determine quickly whether a strain belongs in the latter group, consists in following the uptake of O_2 in the Warburg respirometer with L-alanine and acetate (no oxidation) and with ethanol (oxidation to acetic acid).

The pH optimum for the oxidation of L-alanine by *Gl. oxydans* (*liquefaciens*) was determined.

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THE STEROIDS AND HIGHER FATTY ACIDS OF THE PHYTOFLAGELLATE *POLYTOMA UVELLA*[1]

by

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INTRODUCTION.

The steroids and fatty acids of the phytoflagellates as well as of other protozoa have as yet received little attention. More is known about the steroids of other micro-organisms (yeasts, bacteria) and of representatives of the class *Chlorophyceae*, with which *Polytoma uvella* is thought to be related phylogenetically (FIESER and FIESER, 1959). The absence of steroids in some micro-organisms has been reported (VISHNIAC and WATSON, 1953). Provitamin-D_2 (ergosterol) seems to predominate amongst the steroids of the lower orders of cryptogams (bacteria, yeasts, fungi, brown algae) (BILLS, 1935). So far all other provitamins-D have been found outside the group of the micro-organisms (*cf.* ROSENBERG and WADDEL, 1951). The literature on the fatty acids in *Chlorophyceae* and other classes of higher algae is very scanty (BERLEW, 1953).

In a previous publication we reported on the carotenoids of *Polytoma uvella* (LINKS, VERLOOP and HAVINGA, 1960). The present paper deals with investigations undertaken to extend our knowledge of the lipids and steroids of this organism.

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[1] The experimental work was carried out during the period 1951-1952. A preliminary note appeared in 1954 (LINKS *et al.*, 1954) and a detailed report in the dutch language in 1955 (LINKS, 1955).

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EXPERIMENTAL.

Organism. The culture of *Polytoma uvella* used was designated as "strain 1" by Prof. Dr. E. G. PRINGSHEIM and was obtained through his courtesy from the Type Culture Collection of Algae and Protozoa, Botany School, Cambridge, England.

Medium. Two culture media were used, both containing (% w/w): sodium acetate, 0.21; asparagine, 0.02; glycine, 0.2; glucose, 0.2; K_2HPO_4, 0.02; Na_2CO_3, 0.02; $MgSO_4.7H_2O$, 0.01 in distilled water.

In addition, medium 1 (used in series 1) contained: $FeCl_3$,0.001; $CaCl_2$, 0.001; and thiamin, 0.00013; medium 2 (used in series 2a and 2b) was supplemented with 10% (v/v) yeast extract (made by autoclaving). The pH was adjusted to 7–7.5 before sterilization (20 min. 120° C.). Afterwards it was raised to 7.5–8.0.

The liquid media were inoculated with 1% (v/v) of a five-day old liquid culture grown in the following medium (% w/v): sodium acetate, 0.8; asparagine, 2.0; K_2HPO_4, 0.02; Na_2CO_3, 0.02; $MgSO_4.7H_2O$, 0.01 in distilled water.

Incubation. Cultures were grown in 300 to 1000 ml Erlenmeyer flasks filled to $^1/_3$ – ½ with the medium, at a temperature of 24° ± 1° C., for about 3 weeks; at this time all the flagellates were encysted. Daylight was excluded. The strongly aerobic cultures were shaken by hand daily in artificial light.

Harvesting. At the end of the culture period most of the clear supernatant was removed by suction and the organisms were harvested by centrifugation. The orange-red mass was stored in a polyethylene bottle under N_2 at–10° C.

Yields. Series 1 (150 l) yielded 100 ml wet *Polytoma* cells with a dry weight of 14 g (determined after extraction of the lipids). For series 2a these figures were: 100 l, 80 ml, and 12 g; and for series 2 b: 100 l, 55 ml, and 7.5 g respectively.

Solvents. The solvents employed were spectroscopically pure; purifications were carried out by at least one distillation with a 1 m Widmer fractionation column.

Absolute ethanol, permitting absorption measurements down to 220 mμ was prepared according to VOGEL (1948) and PESTEMER (1951). Benzene, ethyl ether and acetone were purified according to VOGEL (1948). Pure petroleum ether (40°—60° C.) and pure petroleum ether (80°—100° C.; for the paperchromatography) were obtained by oleum treatment and filtration through silica gel (PESTEMER, 1951; GRAFF *et al.*, 1944).

Phenylcellosolve was prepared according to BENTLEY *et al.* (1896). Glycol was purified according to PESTEMER (1951).

Instruments. A Beckman spectrophotometer, model D.U. was employed for the ultraviolet spectra and a model I.R. 2 (single beam) spectrophotometer for the infrared spectra.

Extraction and separation of the carotenoids and the steroids from the fatty acids.

100 ml organisms were suspended in 1 l 12% KOH in methanol saturated with petroleum ether (40°—60° C.) and kept under nitrogen in the dark at room temperature (± 20° C.) for 50 hrs. The procedure followed is presented in Table 1.

Dry weight was determined after centrifugation and an additional extraction of the greyish residue with peroxide-free ether. The methanolic and ethereal extracts were combined in a separatory funnel, and a 5-fold volume of boiled distilled water was added, yielding two layers.

The water layer was further extracted with ether under nitrogen until the extracts were colourless. The combined red ether extracts were washed with distilled water and immediately evaporated to dryness under reduced pressure in a stream of N_2, yielding the "unsaponifiable" fraction (carotenoids and steroids).

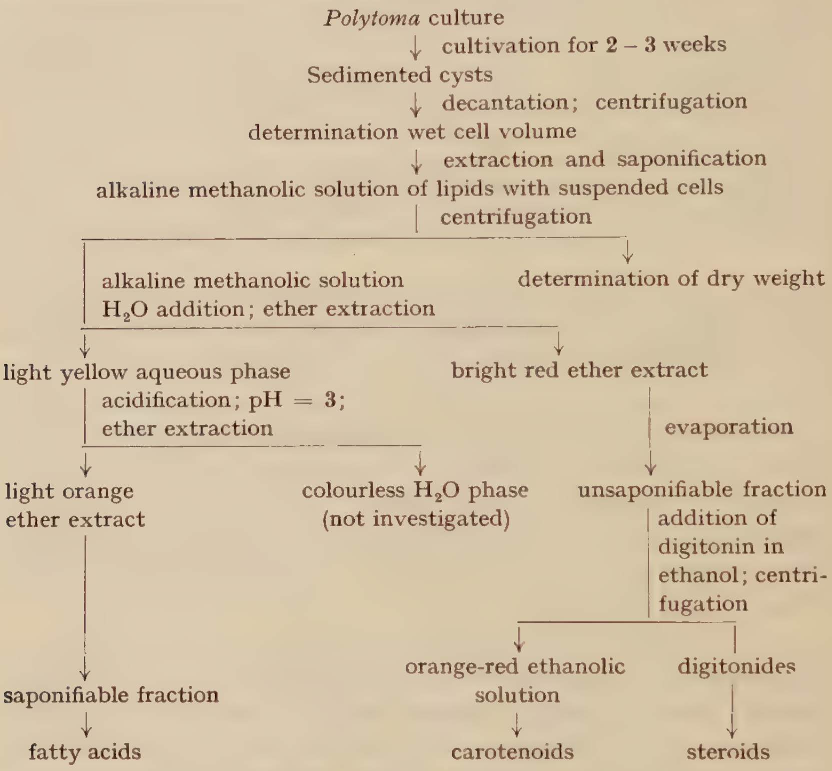
The light yellow water layer was adjusted to pH = 3.5 with 10% HCl, and extracted with ether. Subsequent evaporation of the ether extract yielded a reddish-brown fatty acid fraction (m.p. about 40° C.).

Separation of carotenoids and steroids.

A solution of digitonin in 90% ethanol was added to a solution of the unsaponifiable fraction of series 1 and 2a in absolute ethanol

TABLE 1.

Scheme for the isolation of the lipid soluble material from *Polytoma uvella*.



in the proportion of 7 parts digitonin to 1 part "provitamin D" at room temperature and in artificial light. The suspension was kept under nitrogen at —10° C. for 15 hrs, and centrifuged. The sediment of digitonides was extracted with 90% ethanol and acetone and the extracts were combined with the initial orange-red ethanolic supernatant containing the carotenoids (Table 1).

The digitonides were washed twice with 90% ethanol and once with acetone, and dried over silicagel. The liquids used for washing were discarded. To a solution in the minimum amount of dry pyridine, a tenfold volume of dry peroxide-free ether was added and the suspension was left under nitrogen for 18 hrs. After removing the digitonine by centrifugation and washing the ethereal solution once with 1½% H_2SO_4 and 3 times with distilled water, the ether was taken to dryness under reduced pressure. The resulting weakly pink steroid mixture had a melting range from 120° C. to 140° C. Trial recrystallizations from acetone gave fractions with melting point ranges of several degrees between 135° C. and 162° C.

Paper chromatography of the steroids.

A slightly modified NEHER and WETTSTEIN (1952) procedure was used for the paper chromatographic separation of the steroids. Chromatograms were run at 23° C. as well as at 3° and —8° C. (LINKS, 1955). Whatman no. 1 paper was used. The paperstrips were dried at 85° C. for 2 hours before impregnation. After impregnation they contained about 11% (w/w) phenylcellosolve. The steroids were applied, dissolved in trichloroethylene. The Carr and Price reagent for the colour development was stabilized with acetic anhydride.

Analysis of the fatty acids.

The unsaturated fatty acids were determined spectrophotometrically after alkaline isomerization in glycol according to BRICE *et al.* (1951). Iodine values were determined by means of the method of WIJS (Methods of analysis, Ass. Off. Agr. Chem. 1945. 6th ed. p. 495). The saturated higher fatty acids were determined after the oxidative destruction of the unsaturated fatty acids according to SCHUETTE and NOGARE (1951).

RESULTS AND DISCUSSION.

Analysis of the steroid mixture.

The steroid mixture isolated from *Polytoma uvella* was compared with the provitamins-D, 7-dehydrocholesterol and ergosterol, on paper chromatograms (16 hrs; running distance 15 cm; 23° C.). One single *Polytoma* steroid and 7-dehydrocholesterol moved together and a little faster than ergosterol. The opposite happened in a mobile phase of light petroleum (80—100° C.) with 10% (v/v) benzene.

At —8° C. only ergosterol showed considerable tailing; the same phenomenon was observed at —8° C. with a crude yeast steroid mixture (main component: ergosterol). As we detected no other spot with the Carr and Price reagent, the conclusion may be drawn that other 3β-hydroxy-Δ 5,6-steroids were probably absent. For the amounts and the varying "purity" of the *Polytoma* provitamin-D we refer to table 2.

TABLE 2.

Data on the steroid fractions isolated from *Polytoma* cells.

Series	Volume of wet cells after centrifugation	Dry weight of organisms	Amount of steroids[1])	Content of provitamin-D[2])	
				uncorrected	corrected
1	100 ml	14 g	0.068 g	47%	42%
2a	80 ml	9.5 g	0.027 g	61%	54%
2b	55 ml	7.5 g	0.178 g	20%	17%

[1]) Determined after splitting of the digitonides.

[2]) We assumed: $E^{1\%}_{1\ \text{cm}}$ Polytoma "provitamin D" (282 mμ) =

$E^{1\%}_{1\ \text{cm}}$ ergosterol (282 mμ) = 302

The correction was made by subtracting [E (240 mμ) + E (320 mμ)] from E(282 mμ)

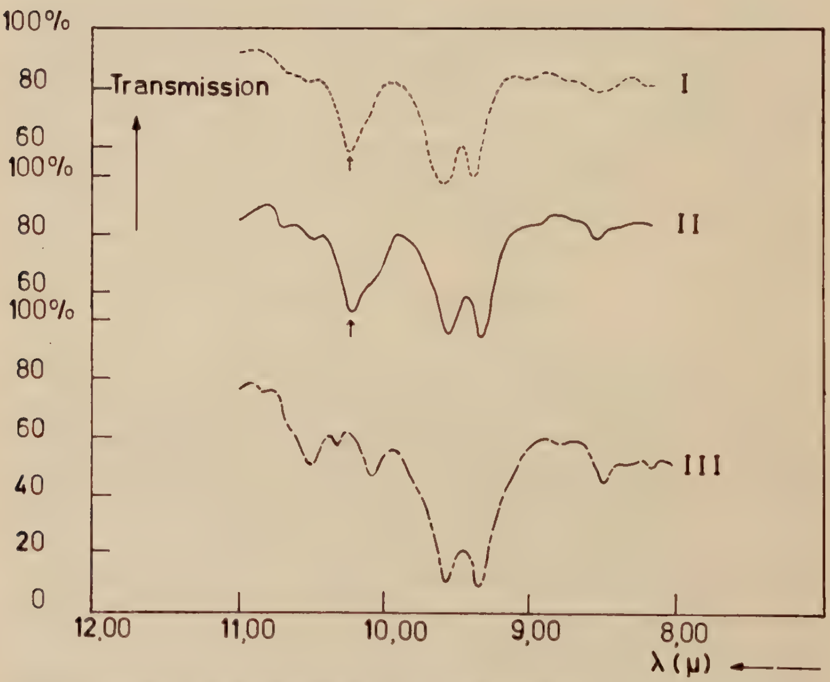


Fig. 1. Infrared absorptionspectra of *Polytoma* steroid mixture (I), ergosterol (II) and 7-dehydrocholesterol (III) in CS_2.

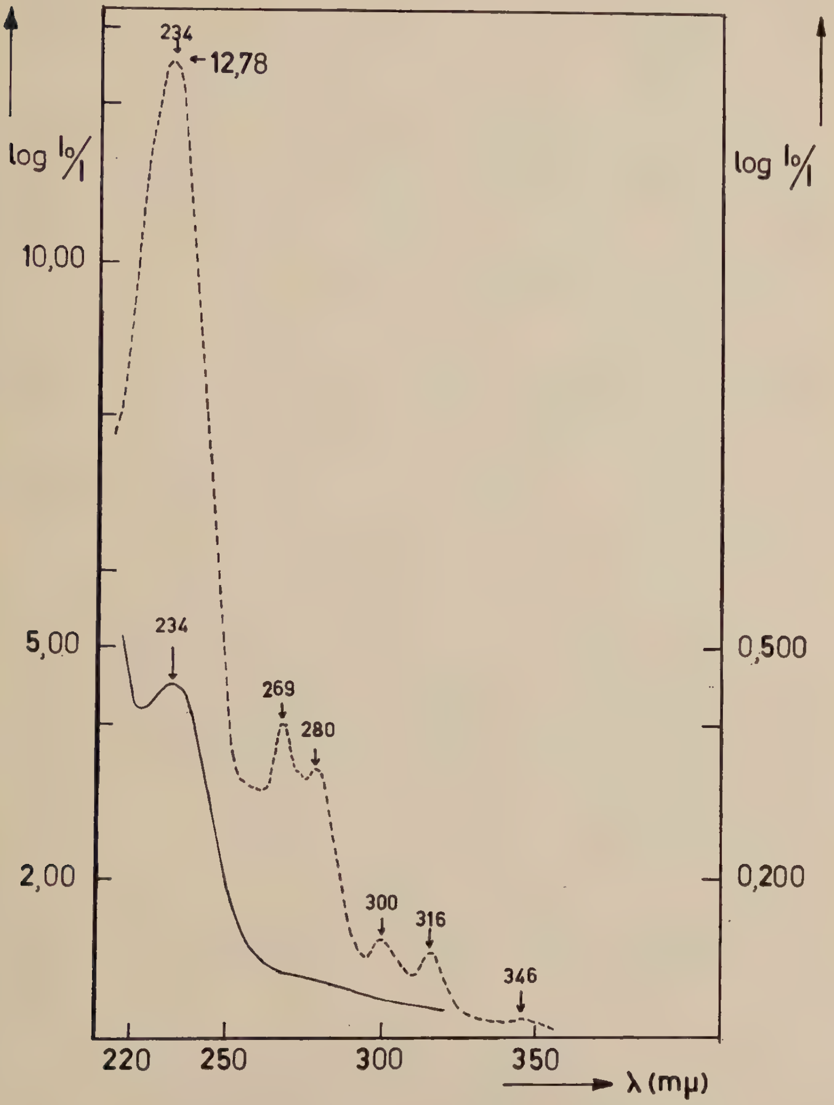


Fig. 2. Ultraviolet absorptionspectra of the higher fatty acids from *P. uvella* in absolute ethanol before (—— right hand side extinction scale) and after (. . . . . left hand side scale) isomerization. The curves before and after isomerization are calculated on the same concentration.

The ultra-violet absorption spectra of the steroid mixtures in light petroleum (40°—60° C.) had their maxima at the same wavelengths as those of the known provitamins-D (*cf.* HOGNESS *et al.*, 1937; HUBER *et al.*, 1945).

Infrared absorption spectra (1—2% in CS_2; 1 mm; NaCl cell) are reproduced in fig. 1. The characteristic absorption (10.28—10.31 μ) of steroids with a trans double bond in the side chain at C-atom 22 was observed with ergosterol as well as with the *Polytoma* steroid mixture (TURNBULL *et al.*, 1950; NORMAN JONES, 1950). The possibility exists that this absorption is due to a component other than the provitamin-D-like component in the mixture. We would like to call attention, however, to the striking similarity of the spectra of ergosterol and the crude *Polytoma* steroid in the whole region 8.80—10.60 μ. The experimental data thus suggest the presence of a steroid, similar to, but not identical with ergosterol or 7-dehydrocholesterol.

Provitamin-D-like substances were found in *Polytoma uvella* irrespective of the N-source, used in cultivation (asparagine, ammonium acetate, glycine or yeast extract).

TABLE 3.

Total amounts of higher fatty acids in *Polytoma uvella.*

Series	Total amount (g)	g/100 ml organisms¹)	g/100 ml dry weight²)	"purity"³) %
1	1.50	1.58	10.7	99.5
2b	0.75	1.38	10.0	99.5

¹) g/100 ml = g/100 g "wet weight".

²) Dry weight amounted to 14.0 and 7.5 g for series 1 and 2b resp. after lipid extraction.

³) "Purities" were determined by titration of 50 mg fatty acid mixture with 0.1 N sodium ethanolate in ethanol; the mol. weight of oleic acid was taken as the mean mol. weight of the mixture.

TABLE 4.

Relative percentages of the higher fatty acids in *Polytoma uvella.*

Series	Saturated	Mono-enoic¹) %	Dienoic (conjugated) %	Dienoic (non-conjugated) %	Trienoic (non-conjugated) %	Tetraenoic (non-conjugated) %
1	22.7	30.7	1.1	21.7	10.0	10.7
2b	33.0		0.5	24.8	11.6	14.3

¹) The iodine addition number was 132 for series 1.

Analysis of the fatty acid mixture.

The quantitive results of the analysis of the *Polytoma* higher fatty acids are represented in tables 3 and 4.

The weak ultra violet absorption at 346 mμ (fig. 2) after isomerization, points to the presence of small amounts ($<3\%$) of pentaenoic and possibly even more highly unsaturated fatty acids. The calculated percentages of dienoic and trienoic acids were at most very slightly influenced by it. The percentage of tetraenoic acid would have to be lowered by maximally 3% of the total.

Summary.

1) The steroid mixture from *Polytoma uvella*, precipitated with digitonine, was investigated spectrophotometrically (ultraviolet and infrared) and by means of paper chromatography at different temperatures.
2) A provitamin-D-like substance, similar but not identical with ergosterol and probably also not identical with 7-dehydrocholesterol was present in a varying percentage (15—55%) of the total steroids.
3) The total fatty acids amounted to 11% of the dry weight. Saturated acids amounted to 23—33%, monoenoic acid to about 30%, dienoic acid to 22—25%, trienoic acid to 10—12% and tetraenoic acid to 11—14% of the fatty acid mixture.

More highly unsaturated acids were present in small amounts only.

Acknowledgements.

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ISO-ALKANE OXIDATION BY A *PSEUDOMONAS*

PART I. — METABOLISM OF 2-METHYLHEXANE

by

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Introduction.

In previous studies (Thijsse and van der Linden, 1958; Heringa *et al.*, 1961) on the oxidation of *n*-alkanes by a strain of *Pseudomonas aeruginosa*, it was found that primary attack on the alkane molecule is on the α-C atom exclusively. Oxidation of a terminal methyl group yielding the fatty acid is followed by β-oxidation. Concomitant oxidation of the α- and ω-C atoms was rejected as a second pathway of degradation, although the argument for this was obtained from multiple adaptation experiments only.

Azoulay and Senez (1960) also investigated a *Pseudomonas* strain and obtained similar results. For further information the reader is referred to Stewart *et al.* (1959), Leadbetter and Foster (1959), and Baptist and Coan (1959).

For branched alkanes we found the oxidation to be low or nil in general (Thijsse *et al.*, 1959). Alkanes substituted with only one methyl group, however, in most instances showed an oxidizability high enough to enable them to be studied. In the present investigation on the oxidation of 2-methylhexane, special attention has been paid to the point of initial attack, which was supposed to be a terminal C atom, either C_1 or C_6. Multiple adaptation methods were used and, besides, intermediate fatty acids were detected by gas-liquid chromatographic techniques (GLC).

Experimental.

Organism. The organism used was *Pseudomonas aeruginosa* (KSLA strain 473), isolated and cultured as described earlier (Thijs-

SE and VAN DER LINDEN, 1958). The respiratory quotient (RQ) of resting, 2-methylhexane grown, cells with this hydrocarbon as a substrate was 0.62, indicating complete oxidation of the substrate to carbon dioxide and water. Oxygen consumption (Q_{O_2}) in μl O_2 per hour per mg bacteria (dry weight) during 2-methylhexane respiration is approximately 75, as compared with a $Q_{O_2} = 160$ during heptane respiration by heptane-grown cells. The Q_{O_2} of endogenous respiration was 15 and remained constant throughout the Warburg experiments which were in general of 90 min. duration.

The organism was grown on mineral agar with the hydrocarbon present in the vapour phase. Larger amounts of the heptane-adapted cells were – for convenience – harvested from a laboratory fermentor (HERINGA *et al.*, 1960).

Chemicals. Hydrocarbons used for growing the micro-organism were of at least 99.0% purity. 2-Methylhexanoic and 5-methylhexanoic acids were synthesized whilst other acids were obtained from commercial sources. Chemicals were purified by distillation if needed. All substrates had a purity of 97% or more by GLC standards.

Multiple adaptation. Multiple adaptation was studied by the determination of oxygen consumption rates of suspected intermediates as described by STANIER (1950), employing Warburg techniques as described by UMBREIT *et al.* (1950). Warburg flasks were filled as follows: Main compartment: 1 ml Sörensen phosphate buffer pH 6.8. Fatty-acid sodium salt solutions 0.1 ml (for concentrations see Table 1). Side arm: 1 ml bacterial suspension in buffer approximating 1.3 mg bacteria (dry weight). Center well: 0.2 ml 10% potassium hydroxide. Total volume 2.1 ml + 0.2 ml. Temperature 30° C.

Fatty acid isolation. Suspensions of 5 g bacteria (wet weight) in 200 ml phosphate buffer pH 6.8 were incubated in a stoppered one l flask for 18 hours at 25° C. in the presence of 0.5 ml 2-methylhexane. The limited amount of oxygen available under these conditions presumably induces accumulation of trace amounts of intermediate fatty acids. The incubation mixture was centrifuged and the supernatant liquid was percolated with ether, first at alkaline then at acid pH value. The dried ethereal fatty acid solution was treated with diazomethane and the methyl ester solution was concentrated by distillation.

Gas liquid chromatography (GLC). The GLC apparatus was provided with a sensitive flame ionization detector the signal of which was fed *via* a "Vibron Flame Ionization Detector Amplifier" (Electronic Instruments, Ltd.) to a 2½ mV recording potentiometer. Hydrogen was used as the carrier gas. A pressure difference of 0.51 atm over the 4 m column (4 mm bore, filled with Sil-O-Cel 30-50 mesh impregnated with 30% polyethylene glycol) gave a gas flow rate of 110 ml per min as measured at the end of the column. The temperature was 100° C.

Peaks were identified by the addition of model compounds to the samples.

RESULTS AND DISCUSSION.

Multiple adaptation experiments.

In *Pseudomonas* cells grown on various *n*-alkanes (or *n*-fatty acids) two terminal respiration systems seem to be involved in the catabolic process. The first one – acetate respiration – is always present. The presence of the second one – propionate oxidation – depends on the molecular structure of the substrate of growth. Its capacity is expected to decrease with increasing chain length of the odd numbered alkanes, as only one propionic acid molecule is formed from the higher as well as from the lower members.

The same probably obtains for cells grown on alkanes with one methyl branch[1]) or monomethyl fatty acids (KENNEDY, 1957). Testing for the presence of terminal propionate respiration thus seems a valuable tool in metabolic studies on alkane or fatty acid oxidation. In this way the *iso*-alkane-grown cells may be compared with *n*-heptane-grown cells with high (adaptive) or *n*-hexane-grown cells with low (constitutive) propionate oxidation capacity (THIJSSE and VAN DER LINDEN, 1958).

If the oxidation of 2-methylhexane proceeds from C_1, the following acids are expected to be among the intermediates (KENNEDY, 1957): 2-methylhexanoic, propionic, butyric and acetic acid. If, however, oxidation starts at C_6, the acids to be expected are: 5-methylhexanoic, *iso*-valeric, β-ketobutyric and acetic acid.

Hence, a conclusion as to the oxidation pathway may well be obtained by studying whether 2-methylhexane-grown cells are adapted to propionate or not. 2-Methylhexanoic, 5-methylhexanoic and *iso*-valeric acids were also investigated. Butyric acid was included in our experiments to test the capacity for terminal acetate respiration.

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[1]) Some structures, *e.g.* 2-methylpentane, may induce *iso*-butyrate oxidation instead of propionate oxidation. These two systems may be closely related via methylmalonate.

By use of two Warburg respirometers, heptane- and 2-methylhexane-grown cells as well as the substrates mentioned above could be incorporated in each experiment. The difference between the experiments is in the substrate concentration and in the bacterial preparation only. Three typical experiments are tabulated in Table 1.

TABLE 1.

Q_{O_2}* of heptane- and 2-methylhexane-grown *Pseudomonas* cells with various fatty-acid substrates**.

Pseudomonas adapted to	Fatty-acid substrate				
	Propionate	*i*-Valerate	2-Methylhexanoate	5-Methylhexanoate	Butyrate
	Expt. No. 1. Substrate conc. 40.5 μ Mol/Flask				
Heptane	118	58	47	47	112
2-Methylhexane	42	100	80	132	156
	Expt. No. 2. Substrate conc. 27.0 μ Mol/Flask				
Heptane	110	57	57	57	80
2-Methylhexane	43	87	75	113	145
	Expt. No. 3. Substrate conc. 13.5 μ Mol/Flask				
Heptane	58	35	36	38	62
2-Methylhexane	55	115	81	160	163

*Q_{O_2} = μl O_2 consumed per hour and per mg bacteria (dry weight).

**Not corrected for endogenous respiration, which was low and constant ($Q_{O_2} = 15$).

The figures demonstrate high propionic acid oxidation by heptane-, and low propionic acid oxidation by 2-methylhexane-grown cells. The figures of Expt. No 3 do not seem convincing but they can be explained by the low propionate concentration, the latter being sub-optimal for heptane-grown cells. Low propionate oxidation by 2-methylhexane-grown cells can be explained by assuming that in 2-methylhexane degradation the C_6 pathway is favoured. This pathway does not induce further development of (constitutive) propionate-oxidizing enzymes.

This conclusion is substantiated by the oxidation rates of *iso*-valeric acid. This acid is an intermediate in the C_6 pathway of 2-methylhexane catabolism, but it does not occur in the C_1-degradation scheme. In addition, it is not an intermediate in heptane oxidation. High oxidizability of *iso*-valeric acid by 2-methylhexane-grown cells thus again points to a preference of the micro-organism for primary attack on C_6.

Neither 2-methylhexanoic acid nor 5-methylhexanoic acid are intermediates in heptane oxidation. Consequently, their oxidation by heptane-grown cells is slow. The fact that 5-methylhexanoic acid is oxidized at a higher rate than the 2-methyl derivative by 2-methylhexane-grown cells again indicates the preference of the micro-organism for a degradation scheme *via* a primary oxidation of C_6.

Butyric acid oxidation (acetate respiration) seems lower in heptane- than in 2-methylhexane-grown cells. This is not surprising as the terminal respiration system of the heptane-grown cells is not fully employed – part of it being reserved for propionic acid metabolism – when butyrate is the substrate. In 2-methylhexane-grown cells – assuming C_6 oxidation – the acetate respiration system will be more developed as the 2-methylhexane molecule is probably degraded *via* acetate only.

Summarizing the results of the multiple adaptation experiments we can say that in 2-methylhexane degradation by 2-methylhexane-grown cells the pathway via 5-methylhexanoic and *iso*-valeric acid is clearly favoured. The appreciable oxidation of 2-methylhexanoic acid might indicate that oxidation via C_1 does occur to some extent. However, the conclusion that this second pathway is actually followed in the 2-methylhexane respiring cell is not warranted on the basis of multiple adaptation experiments only.

Isolation and identification of fatty-acid intermediates.

In preliminary experiments on fatty-acid isolation, heptane-grown cells were incubated with 2-methylhexane. From these experiments we learned that 2-methylhexanoic acid, as well as the 5-methyl isomer are formed (Fig. 1). In addition *iso*-valeric acid could be identified in the chromatogram. When, however, 2-methylhexane-grown cells were used in a similar experiment, the amount of 5-methylhexanoic acid as compared with the 2-methyl derivative decreased. Moreover, *iso*-valeric acid disappeared. (Fig. 2).

These results are in good agreement with the Q_{O_2} values quoted in Table 1. A high oxygen consumption will decrease the accumulation

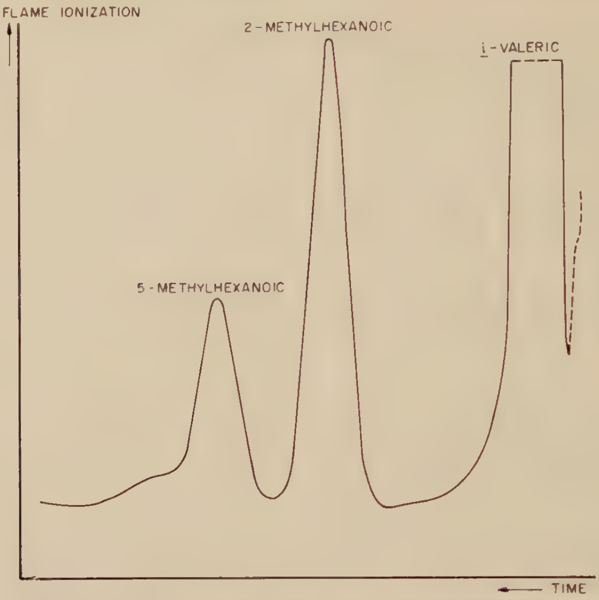

Fig. 1. Chromatogram of fatty acids from 2-methylhexane incubation with heptane-grown *Pseudomonas* cells.

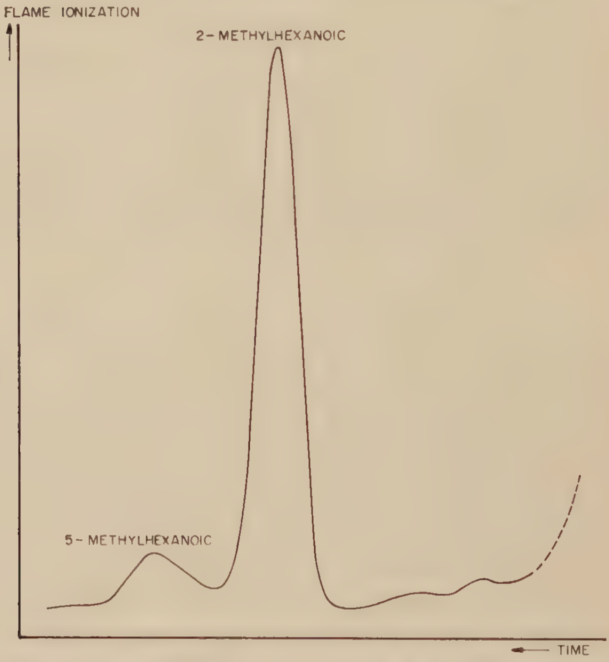

Fig. 2. Chromatogram of fatty acids from 2-methylhexane incubation with 2-methylhexane-grown *Pseudomonas* cells.

of the particular fatty acid intermediate, whilst low Q_{O_2} values will favour the detection. Thus, inspection of Table 1 learns that – using heptane-grown cells – *iso*-valeric, 2-methylhexanoic and 5-methylhexanoic acids may well be accumulated and these acids were in fact identified.

If 2-methylhexane-grown cells are used, 2-methylhexanoic and propionic acids are expected from inspection of Table 1 to accumulate. 2-Methylhexanoic acid was indeed identified, but propionic acid, unfortunately, could not be detected among a number of unknown peaks in the beginning of the chromatogram. The small size of the peak of 5-methylhexanoic acid in this chromatogram (Fig. 2) is also in agreement with its Q_{O_2} value.

Apparently C_1-oxidation, as well as C_6-oxidation do actually occur. However, if 2-methylhexane-adapted cells are used, C_1-oxidation seems to be of minor importance as compared with a degradation scheme *via* C_6, comprising 5-methylhexanoic and *iso*-valeric acids.

Combining our results from multiple adaptation and isolation experiments we can say that some 2-methylhexane is actually oxidized via C_1 in 2-methylhexane-grown cells, though this pathway seems only of minor importance. Heptane-grown cells show less specificity and here probably both types of 2-methylhexane degradation operate to a comparable, but low, extent.

Alkane catabolism and propionate oxidation.

In an earlier paper (THIJSSE and VAN DER LINDEN, 1958), dealing with the catabolism of *n*-alkanes, we have shown that propionate is oxidized at high rates by heptane-grown cells as compared with the hexane-grown micro-organism. In later work (HERINGA *et al.*, 1961) propionic acid was isolated from heptane cultures (6.3% of total fatty acids), whereas its amount in hexane-grown cultures was always low (0.3%). This was in line with expectations, as propionic acid is presumed to be an intermediate in heptane, but not in hexane degradation. In the present study, our conclusions are again based, at least partly, on propionate oxidation rates.

In a recent study on the oxidation of *n*-alkanes AZOULAY and SENEZ (1960) used chloramphenicol to suppress any secondary adaptation during the Warburg experiments. Their results, in general, agree with our experiments, but they differ in some important details, *e.g.* in propionic-acid oxidation rates of hexane- and heptane-grown cells.

Their results suggest that propionic acid is not an intermediate in heptane catabolism and thus clash with our concept of *n*-alkane oxidation. We felt obliged therefore to re-investigate propionate oxidation with the utmost care in the presence of 250μg/ml chloramphenicol (50μg/ml completely inhibits protein synthesis). No appreciable effects of this addition were encountered in experiments of 90 min duration as can be seen from Table 2.

TABLE 2.

Q_{O_2} of Propionate oxidation.

Ps. 473 adapted to	Heptane	Hexane
Chloramphenicol absent	116	33
Chloramphenicol present	108	33

We have also occasionally used chloramphenicol in the other experiments, but no effects were encountered if substrates of high purity were used and therefore we feel safe in our conclusion that secondary adaptations did not interfere with previous and present experiments.

Another point to be mentioned is that according to Azoulay and Senez (1960) *n*-alkane-grown cells show specific action with regard to the chain length of fatty acid substrates. They demonstrated, for example, that hexanoic acid – in the presence of chloramphenicol – is not oxidized by heptane-grown cells. However, in our hands, this and similar experiments gave results that checked well with those of previously published ones without chloramphenicol (Thijsse *et al.*, 1958), in which no such specificity was found. Low specificity of our strain with respect to the fatty acids of medium chain length thus appears well established.

Summary.

Pseudomonas cells adapted to 2-methylhexane oxidize 5-methylhexanoic and *iso*-valeric acids rapidly. The oxidation rates of 2-methylhexanoic and propionic acids are appreciably lower. It can be concluded that primary attack on the C_6 atom is distinctly favoured over that on C_1.

By use of gas-liquid chromatography, 2-methylhexanoic, 5-methylhexanoic and *iso*-valeric acids were shown to be formed when

heptane-grown cells were incubated with 2-methylhexane. When 2-methylhexane-grown cells were used the amount of 5-methylhexanoic acid decreased compared with the amount of the 2-methyl isomer. Moreover, *iso*-valeric acid could not be detected.

The results make it probable that degradation of 2-methylhexane *via* C_6, comprising 5-methylhexanoic and *iso*-valeric acid, is accompanied by a second pathway via 2-methylhexanoic acid. The latter pathway is of minor importance, in particular in 2-methylhexane-adapted *Pseudomonas* cells.

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THE SENSITIVITY OF MIXED POPULATIONS OF BACTERIA TO INHIBITORS

I. THE MECHANISM BY WHICH *DESULFOVIBRIO DESULFURICANS* PROTECTS *PS. AERUGINOSA* FROM THE TOXICITY OF MERCURIALS[1]

by

A. G. BACHENHEIMER and **E. O. BENNETT**

(Received December 21, 1960).

INTRODUCTION.

The literature concerning the sensitivity of bacteria to antimicrobial agents reveals that the effectiveness of most inhibitors is measured in terms of their ability to kill pure cultures of microorganisms under highly artificial laboratory conditions. In actual practice, inhibitors are generally employed against a mixed microbial flora rather than pure cultures.

Recently, BENNETT and BAUERLE (1960) reported that one species may exhibit a marked change in its sensitivity to an inhibitor when another organism is present in the environment. These workers studied the sensitivities of pure and mixed cultures of *Pseudomonas aeruginosa* and *Desulfovibrio desulfuricans* to phenols and mercurials. It was shown that higher concentrations of phenols were necessary to inhibit pure cultures of *Ps. aeruginosa* than mixed cultures and that greater concentrations of phenols were required to inhibit the sulfate-reducers in mixed cultures than in pure cultures. The concentrations of mercurials necessary to inhibit pseudomonads were greatly increased when sulfate-reducing bacteria were present in the environment.

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[1] This investigation was supported by a grant from the Johnson's Wax Fund, Racine, Wisconsin, U. S.A.

The objective of this investigation was to determine the mechanism by which *D. desulfuricans* protects *Ps. aeruginosa* from the inhibitory activity of phenylmercuric lactate.

METHODS.

The same strains of *Ps. aeruginosa* and *D. desulfuricans* used by BENNETT and BAUERLE (1960) were employed throughout this investigation. The cultures were transferred and examined for purity once each month. The sulfate-reducers were examined as described by GUYNES and BENNETT (1959), and the pseudomonads were studied for purity using routine bacteriological procedures. The stock cultures were grown in a semi-solid medium (Medium A) composed of:

Na_2SO_3	0.1 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.2 g
$(NH_4)_2SO_4$	1.0 g
$Fe(NH_4)(SO_4)_2.12H_2O$	0.1 g
Ascorbic acid	0.1 g
Peptone (Difco)	1.0 g
Yeast extract	1.0 g
Agar	3.0 g
Sodium lactate (60%)	7.0 ml
pH 7.6 phosphate buffer	100.0 ml
Distilled water	900.0 ml

(final pH after autoclaving 7.0)

Inocula were prepared for each experiment by inoculating 160 ml of medium A in screw-capped bottles with 1 ml of the stock cultures. The cultures containing pseudomonads were incubated for 24 hours and the *Desulfovibrio* cultures for 4 days at 30° C. The cell suspensions were shaken vigorously before use to insure uniform suspensions, and were of sufficient quantity to inoculate all of the tubes in each experiment.

A stock solution of phenylmercuric lactate was prepared by dissolving 1 g of inhibitor in 100 ml of distilled water. The different concentrations of inhibitor were prepared by dispensing 1 ml of varying dilutions of the stock solution into chemically clean screw-cap test tubes containing sufficient medium to make final volumes of 9 or 19 ml. The mercurial concentration ranged upwards from 50 ppm in integrals of 50 ppm. The tubes were autoclaved and checked for possible contamination by incubating them for 16 hours at room temperature.

The pure cultures were prepared by adding 1 ml of inoculum to the tubes containing 9 ml of medium and inhibitor. Mixed cultures were prepared by inoculating 1 ml each of the *D. desulfuricans* and *Ps. aeruginosa* cultures into tubes containing 18 ml of medium. The tubes were incubated for 48 hours at 30° C., shaken vigorously to assure uniform suspensions, and then 0.5 ml from each tube containing pseudomonads was transferred into 5 ml of thioglycollate broth. The tubes containing *Desulfovibrio* were subcultured into medium A containing 3.75 g/l of sodium thioglycollate. The subcultures were made in duplicate and were used to determine the inhibitory concentration of each organism. The viability of the sulfate-reducers was determined by the production of hydrogen sulfide in the subculture medium. The viability of the pseudomonads was determined by the production of turbidity in the thioglycollate broth and microscopic examination. The inhibitory concentration was established as the lowest concentration of the inhibitor which completely prevented growth in the subcultures.

In certain instances, *D. desulfuricans* was grown in a sulfate-free medium described by POSTGATE (1952) and sulfide concentrations were determined in some experiments by using the quantitative method of POMEROY (1944).

In certain experiments, hydrogen sulfide or ferrous sulfide was added to the medium in place of ferric ammonium sulfate. A hydrogen sulfide generator was fitted with sterile glass tubing and a glass-wool filter. Hydrogen sulfide was bubbled into the sterile medium; the sulfide concentration was determined, and dilutions were made using additional quantities of sterile medium. The diluted medium was again analyzed for sulfide content and then aseptically added to tubes containing sterile dilutions of inhibitor. Known amounts of finely ground ferrous sulfide were suspended in the medium and the tubes were autoclaved at 15 pounds steam pressure for 15 minutes. Several tubes of each concentration were analyzed for their sulfide concentration before and after autoclaving and the results showed that the ferrous sulfide was evenly distributed and unaffected by autoclaving. The tubes containing 19 ml of sulfide medium and inhibitor were inoculated with 1 ml of a suspension of pseudomonads. All experiments were done in duplicate and repeated several times.

RESULTS.

The data in Table 1 show that *Ps. aeruginosa* is more difficult to inhibit with phenylmercuric lactate in the presence of *D. desulfuricans*. Replicate determinations of the inhibitory concentrations were different from one experiment to another, but the trend was consistently the same.

TABLE 1.

The inhibitory activity of phenylmercuric lactate against pure and mixed cultures of *Ps. aeruginosa* and *D. desulfuricans*.

Inhibitory concentration (ppm)		
Ps. aeruginosa		*D. desulfuricans*
Pure culture	Mixed culture	Pure culture
100	900	900
200	1000	900
200	1000	900
200	900	800
300	800	800
400	1000	800
200	900	900
100	1000	700
200	800	500
100	900	700

The next step involved determining whether the protective substance was associated with the *Desulfovibrio* cells or whether it was present in the supernate. In this experiment, the sulfate-reducers were grown in medium A with no agar in order to facilitate the removal and washing of the cells. The washed cells were prepared by centrifuging a culture at 5,000 rpm for 30 minutes, washing the cells three times with physiological saline and resuspending in saline to the original volume. The supernate from the culture was filtered through an ultra-fine fritted glass filter to remove any cells that might have remained after centrifugation. The sulfate-reducers also were grown in the sulfate-free medium developed by POSTGATE (1952).

Table 2 shows that the cultures containing the highest concentrations of sulfide offered the most protection to the pseudomonads. It may be noted that the sulfide concentration in POSTGATE's medium

TABLE 2.

The inhibitory activity of phenylmercuric lactate against *Ps. aeruginosa* in the presence of different *D. desulfuricans* culture inocula.

D. desulfuricans culture fraction	Sulfide content (ppm)	Inhibitory concentration for *Ps. aeruginosa* (ppm)
None	0	200
Washed cells	5	200
Cell-free supernate	80	600
Normal inoculum (Medium A)	300	1000
Normal inoculum (POSTGATE's medium)	11	200

The washed cells of *D. desulfuricans* were inhibited by 20 ppm.

The pure culture of *D. desulfuricans* grown in medium A was inhibited by 900 ppm.

The pure culture of *D. desulfuricans* grown in POSTGATE's medium was inhibited by 100 ppm.

was very low; however, the number of cells/ml was only approximately 15% less than in medium A. It may be observed that the pseudomonads were not protected by the sulfate-reducers in this medium since the sensitivity of the latter organisms was less than that needed to inhibit the pseudomonads.

These studies definitely implicated sulfides as the factors responsible for the protective effect. The next series of experiments was designed to prove that the sulfides were responsible for the protective effect and to determine the relative importance of the dissolved and precipitated sulfides in the process.

Medium A contained ferric ammonium sulfate which combines with hydrogen sulfide to form insoluble iron sulfide. Quantitative sulfide analysis of *Desulfovibrio* cultures containing varying amounts of ferric ammonium sulfate revealed that all of the cultures had the same concentration of sulfide. However, as the concentration of the iron salt was increased, the concentration of hydrogen sulfide decreased and the concentration of iron sulfide increased.

The precipitated sulfide was far less effective in protecting *Ps. aeruginosa* from the inhibitory action of the mercurial than the dissolved sulfide. This was shown for mixed cultures containing various amounts of ferric ion (Table 3) and also for pure cultures of *Ps.*

aeruginosa, to which various amounts of hydrogen sulfide and ferrous sulfide had been added (Table 4).

TABLE 3.

The effect of ferric ammonium sulfate on the sensitivity of mixed cultures to phenylmercuric lactate.

Concentration of ferric ammonium sulfate (g/l)	Inhibitory concentration (ppm)		
	Ps. aeruginosa		*D. desulfuricans*
	Pure	Mixed	Pure
0.00	200	>1,000	>1,000
0.05	200	1,000	1,000
0.10	200	800	900
0.15	200	700	800
0.20	300	700	700

TABLE 4.

The effect of ferrous sulfide and hydrogen sulfide on the inhibitory activity of phenylmercuric lactate against *Ps. aeruginosa.*

Sulfide concentration (ppm)	Inhibitory concentration (ppm) in presence of ferrous sulfide	Inhibitory concentration (ppm) in presence of hydrogen sulfide
0	200	200
5	200	400
10	200	500
15	200	600
20	200	600
40	200	700
80	200	>1000

Mean values of three different duplicate experiments.

Thus, it appears that *D. desulfuricans* can protect *Ps. aeruginosa* from the inhibitory activity of phenylmercuric lactate due to the production of hydrogen sulfide.

DISCUSSION.

It has been observed repeatedly that *Desulfovibrio* is often associated with other bacteria in nature. Many investigators such as

HEUKELEKIAN (1948), KIMATA *et al.* (1955a, 1955b), BUTLIN *et al.* (1956), GUYNES and BENNETT (1959), and ISENBERG and BENNETT (1959) have observed these relationships between sulfate-reducers and other bacteria. To date, there has been no definite information published as to the precise nature of these associations. It has been suggested that the associated organisms benefit *Desulfovibrio* by lowering the oxidation-reduction potential, by providing essential metabolites, and by oxidizing toxic compounds present in the environment. None of these reports indicate that *Desulfovibrio* may be beneficial to the other organisms in the environment.

The report by BENNETT and BAUERLE (1960) and the results of this investigation clearly show that the relationship is reciprocal in nature. The associated organisms may furnish sulfate-reducers with essential metabolites and adjust the oxidation-reduction potential. In turn, the *Desulfovibrio* can protect these associated organisms from the harmful effect of mercurials. It is quite possible that sulfate-reducing bacteria protect associated organisms against many other types of inhibitors since it is well known that they are difficult to control with antimicrobial agents.

It is obvious from the results of this investigation that hydrogen sulfide produced by the *Desulfovibrio* protects *Ps. aeruginosa* from the inhibitory activity of phenylmercuric lactate. The sulfide may be active in two ways to protect the pseudomonads from the mercurial. First, it is known that a mercurial has a greater affinity for hydrogen sulfide than it does for the bacterial cell. Therefore, as shown by McCALLA (1940), the hydrogen sulfide can remove the mercurial from the environment so that the organism never comes in contact with the inhibitor. Secondly, it is known that the antimicrobial activity of a mercurial involves a two phase process. In the first phase, the mercurial is adsorbed by the cells and growth is inhibited; however, the cells are not dead, nor are they damaged beyond repair. It is possible to reverse the effect of the mercurial by precipitation of the Hg-ions during this phase. Hydrogen sulfide has been shown to be one of several compounds capable of this type of neutralization by such workers as ENGELHARDT (1922), STEARN and STEARN (1928), and KRUEGER and BALDWIN (1934). It is obvious from these observations that even if the mercurial is adsorbed by the pseudomonads, the sulfate-reducers can furnish an antidote to neutralize the effect of the inhibitor.

This protective effect is possibly active until the concentration of the mercurial approaches that which is inhibitory for the sulfate-reducers. It may be noted that the inhibitory concentration for the mixture is quite similar to that required to inhibit the pure culture of *D. desulfuricans.*

Pseudomonads and sulfate-reducing bacteria are commonly found together in many different types of microbial problems encountered in the petroleum industry. These organisms are found in water flood systems (CARLSON and BENNETT, 1960), in the environment surrounding pipelines used to transport petroleum (HARRIS, 1960), and in refined petroleum products (GUYNES and BENNETT, 1959). These organisms are believed to be involved in the problems which are encountered in these environments. It is obvious that the relationship between these two organisms and others must be fully understood before effective control of growth can be accomplished.

Summary.

The concentration of phenylmercuric lactate required to inhibit *Pseudomonas aeruginosa* was greatly increased in the presence of *Desulfovibrio desulfuricans.* The concentration of mercurial necessary for inhibition of both organisms was found to be approximately the same as that needed to inhibit the pure culture of sulfate-reducers.

The effect of sulfides on the activity of the inhibitor was studied and related to the sulfide concentration of *Desulfovibrio* cultures. The results indicate that the hydrogen sulfide produced by the sulfate-reducers protects both organisms from the inhibitory activity of the mercurial.

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CLASSIFYING YEASTS ON PUNCH CARDS

by

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(Received January 15, 1961).

Filing taxonomic information so it can be retrieved quickly is a major problem. Placing the data in a book does not fulfill the requirement, especially when new material is published every week and new editions of books are published every few years. No system currently available permits quick location of data common to several species. An answer to the problem is the filing of taxonomic data on punch cards.

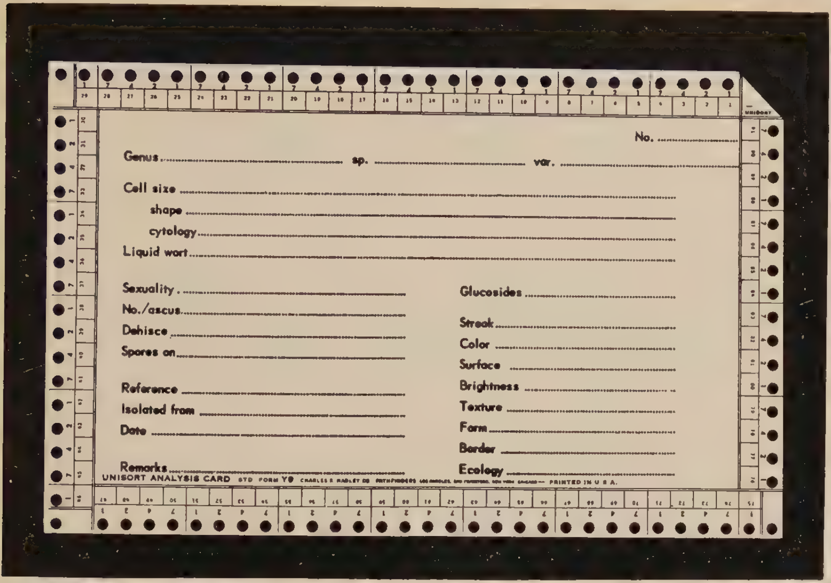
METHOD.

Punch cards are available in a variety of sizes from different manufacturers. Our preference is an 8 × 11-inch card with 91 peripheral, numbered holes, divided into 22 sections, each with 4 holes, plus 1 additional hole on each of three sides of the card. A printed digit directly below each hole gives it a numerical value. These are not to be confused with the numbers below them, which represent the number of the hole. Each digit in a section can be used singly or in combination to obtain any number from 1 to 10, *e.g.*, 2 and 4 represent 6. Digits in the following section represent multiples of ten, *e.g.*, 2 in this section represents 20. By this "indirect method" any number from 1 to 99 can be indicated by proper notching of these 8 holes. In the "direct method" each numbered hole represents one subject. A notched hole means that the character represented by the number of that hole is positive. Whenever a property is weak or variable, the letters "W" or "V" are written below the notched hole. Notched cards are separated from a stack by inserting a long needle through the desired hole; the notched cards fall out and the rest of the cards remain suspended on the needle.

On the face of the card we have printed headings for certain characteristics (fig. 1) not referred to by the peripheral holes. The back of the card is used for drawings of cells, or for typing any additional information.

Following is a description of a possible model, modifiable to suit the needs of the individual:

NUMBER OF HOLE	SUBJECT	COMMENTS
1, 2, and 3	Spore shape 1) round 2) hat 3) kidney 4) oval 5) saturn 6) elongate 7) ballistospore	Indirect method
4	Spore	Direct method
5 — 10	Fermentation 5) glucose 6) galactose 7) sucrose 8) maltose 9) lactose 10) raffinose	Direct method
11 — 15		Open
16	Color	Refers to color of streak
17	NO_3	Ability to utilize nitrate as sole nitrogen source
18	Starch	Ability to produce starch
19	Acid	Production of acid from sugar
20	Vitamin free	Ability to grow in medium without vitamins
21 — 28	Ecology 1) virgin soils 2) cultivated soils 3) oceans 4) lakes 5) high sugar media 6) high salt media 7) high temperature 8) low temperature 9) mammals 10) rodents etc.	Indirect method
29		Open
30	Mycelium	



No.

Genus sp. var.

Cell size

shape

cytology

Liquid wort

Sexuality

No./ascus

Dehisce

Spores on

Reference

Isolated from

Date

Remarks

Glucosides

Streak

Color

Surface

Brightness

Texture

Form

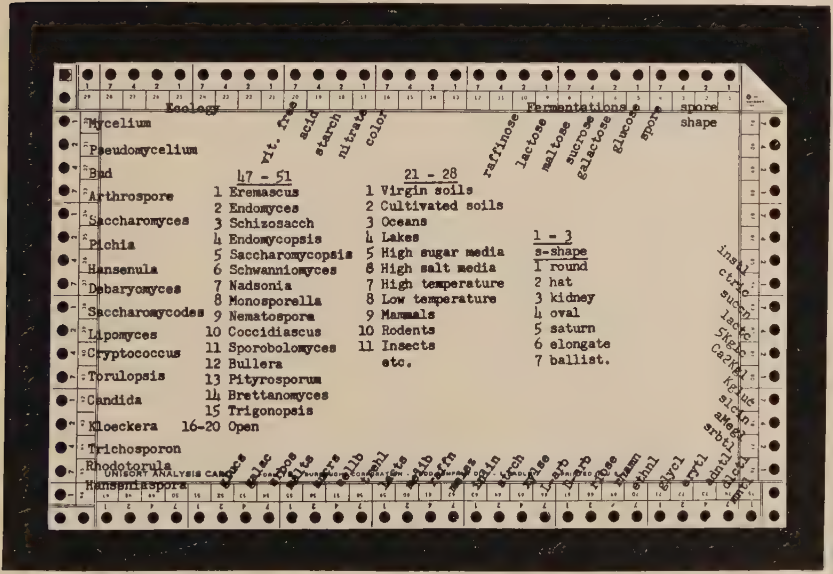
Border

Ecology

UNISORT ANALYSIS CARD

PRINTED IN U S A.

Fig. 1. A punch card with headings for characteristics not used in peripheral coding.



Ecology

Mycelium
Pseudomycelium
Bud
Arthrospore
Saccharomyces
Pichia
Hansenula
Debaryomyces
Saccharomycodes
Lipomyces
Cryptococcus
Torulopsis
Candida
Kloeckera
Trichosporon
Rhodotorula
Hanseniaspora

47 - 51

1 Eremascus
2 Endomyces
3 Schizosacch
4 Endomycopsis
5 Saccharomycopsis
6 Schwanniomyces
7 Nadsonia
8 Monosporella
9 Nematospora
10 Coccidiascus
11 Sporobolomyces
12 Bullera
13 Pityrosporum
14 Brettanomyces
15 Trigonopsis
16-20 Open

21 - 28

1 Virgin soils
2 Cultivated soils
3 Oceans
4 Lakes
5 High sugar media
6 High salt media
7 High temperature
8 Low temperature
9 Mammals
10 Rodents
11 Insects
etc.

1 - 3

s-shape

1 round
2 hat
3 kidney
4 oval
5 saturn
6 elongate
7 ballist.

Fig. 2. Index for classifying yeasts on a punch card.

NUMBER OF HOLE	SUBJECT	COMMENTS
31	Pseudomycelium	
32	Buds	
33	Arthrospores	
34 — 46	Genera (used frequently)	Direct method
47 — 51	Genera	Indirect method
52 — 85	Carbon sources	34 compounds used by WICKERHAM
86 — 91		Open

Fig. 2 shows this sample model on an 8 × 11-inch punch card.

As can be seen from the above, in the direct method each hole represents one subject; the common characteristics or most frequently encountered genera each get a hole. In the indirect method the numbered holes, used singly or in combination, refer to an index that gives the characteristic. It is advisable to leave some holes unused for later inclusion of material forgotten or new.

DISCUSSION.

The advantages of this method are: it offers greater speed in both filing and retrieving information; cards need not be filed in order; it is easy to record data by a notch; cards of yeasts with common characteristics can be easily retrieved; drawings or photographs can be placed on the back of the card.

Should a standard system of recording classification be adopted by yeast taxonomists, taxonomic data could easily be transmitted from one laboratory to another by a card.

Summary.

Attention is drawn to the advantages of punch cards for filing taxonomic information. A model system adapted to yeast taxonomy is presented.

(From the National Institute of Public Health, Utrecht, the Netherlands).

FOUR NEW *SALMONELLA* TYPES (*S. TILBURG, S. BUNNIK, S. OVERSCHIE* AND *S. STERRENBOS*) ISOLATED IN THE NETHERLANDS

by

E. H. KAMPELMACHER and **P. A. M. GUINÉE**

(Received November 28, 1960).

S.tilburg was isolated from the mesenterial lymphnodes of a swine by the authors. *S.bunnik* was isolated by P. ZWART, Institute of Tropical and Protozoan Diseases, State University, Utrecht, from the organs of a reptile (*Basiliscus vittatus*). *S. overschie* was isolated by Dr. J. HUISMAN, Municipal Health Laboratory, Rotterdam, from a tortoise. *S.sterrenbos* was isolated by Dr. J. F. COSTER, Bacteriological and Serological Laboratory, National Institute of Public Health, Utrecht, from the lymphnodes of a patient after an operation.

All the strains showed the typical biochemical behaviour of the genus *Salmonella*. In table 1 the biochemical data are shown.

In table 2 and 3 the serological data are given for the four new *Salmonella* types.

After absorption of O-serum *S.bunnik* with *S.berkeley* a rest titer of 1 : 320 remained. Absorbed with *S.milwaukee* or *S.ahutza* or *S.farcha* or *S.mbao* the same rest titer remained. All these absorbed sera gave no agglutination with all known *Salmonella* and *Arizona* O-antigens. Prof. Dr. F. KAUFFMANN told us that *S.sterrenbos* had a third fase, which was strongly agglutinated by H j-serum.

TA

Biochemical behaviour of *S. tilb*

	glucose + gas	mannitol	adonitol	dulcitol	inositol	lactose	salicine	sucrose	indol	T.S.I.	ammoniumcitrate	methylred	Voges-Proskauer
S. tilburg	++	+	—	+	+	—	—	—	—	+	+	+	—
S. bunnik	++	+	—	+	—	—	—	—	—	+	+	+	—
S. overschie	++	+	—	+	—	—	—	—	—	+	+	+	—
S. sterrenbos	++	+	—	+	—	—	—	—	—	+	+	+	—

Therefore the new *Salmonella* types have the following antigenic formulas:

S.tilburg 1,3,19; d : 1,w
S.bunnik 43 ; z42 : -
S.overschie 51 ; 1,v : 1,5
S.sterrenbos 6,8 ; d : enx

We are indebted to Prof. Dr. KAUFFMANN for confirmation of our findings.

TABLE 2.

Control of O-antigens of *S. tilburg*, *S. bunnik*, *S. overschie* and *S. sterrenbos*.

serum	antigens	
	S. tilburg	*S. senftenberg*
S. tilburg	1 : 2500	1 : 5000
S. tilburg absorbed with *S. senftenberg*	—	—
	S. bunnik	*S. berkeley*
S. bunnik	1 : 640	1 : 320
S. bunnik absorbed with *S. berkeley*	1 : 320	—
	S. overschie	*S. treforest*
S. overschie	1 : 640	1 : 640
S. overschie absorbed with *S. treforest*	—	—
	S. sterrenbos	*S. bovis morbificans*
S. sterrenbos	1 : 2500	1 : 2500
S. sterrenbos absorbed with *S. bovis morbificans*	1 : 80	1 : 80

unnik, S. *overschie* and S. *sterrenbos*.

urea	phenyl-alanine	malonate	lysine decarboxylase	KCN	arabinose	rhamnose	trehalose	xylose	glycerol (Stern)	d-tartrate	l-tartrate	i-tartrate	citrate	mucate	maltose	sorbitol	gelatin
—	—	—	+	—	+	+	+	+	+	+	+	+	+	+	+	+	—
—	—	—	+	—	+	+	+	+	+	—	+	—	+	+	+	+	+
—	—	—	+	—	+	+	+	+	+	+	+	—	+	+	+	+	—
—	—	—	+	—	+	+	+	+	+	+	+	—	+	+	+	+	—

TABLE 3.

Control of H-antigens of *S. tilburg*, *S. bunnik*, *S. overschie* and *S. sterrenbos*.

serum	antigens		
	S. tilburg	*S. muenchen*	*S. dar-es-salam*
S. tilburg	> 1 : 1280	> 1 : 1280	> 1 : 1280
S. tilburg absorbed with *S. muenchen*	> 1 : 1280	—	1 : 640
S. tilburg absorbed with *S. dar-es-salam*	1 : 1280	> 1 : 1280	—
	S. bunnik	*S. bloemfontein*	
S. bunnik	> 1 : 1280	> 1 : 1280	
S. bunnik absorbed with *S. bloemfontein*	1 : 40	—	
	S. overschie	*S. shangani*	*S. potsdam*
S. overschie	> 1 : 1280	> 1 : 1280	> 1 : 1280
S. overschie absorbed with *S. shangani*	1 : 1280	1 : 20	1 : 1280
S. overschie absorbed with *S. potsdam*	1 : 640	1 : 320	—
	S. sterrenbos	*S. muenchen*	*S. abortus bovis*
S. sterrenbos	> 1 : 1280	> 1 : 1280	1 : 640
S. sterrenbos absorbed with *S. muenchen*	1 : 1280	1 : 80	[1]
S. sterrenbos absorbed with *S. abortus bovis*	> 1 : 1280	[1]	—

[1]) not done

Summary.

Four new *Salmonella* types isolated in the Netherlands are described. *S.tilburg*, 1,3,19; d : l, w, was isolated from the lymphnodes of a swine. *S.bunnik*, 43; z42:-, was isolated from a reptile. *S.overschie*, 51; l,v : 1,5, was isolated from a tortoise. *S.sterrenbos*, 6,8; d : enx, was isolated from the lymphnodes of a patient.

(Department of Food Science and Technology, University of California, Davis, California, U.S.A.).

ENDOMYCOPSIS SCOLYTI, A NEW HETEROTHALLIC SPECIES OF YEAST

by

H. J. PHAFF and **M. YONEYAMA** [1])

(Received January 4, 1961).

An extensive survey of the yeast flora associated with bark beetles of the genus *Scolytus*, parasitic on fir (*Abies*) and Douglas fir (*Pseudotsuga*), revealed a heterothallic yeast which was present in significant numbers and of wide geographic distribution. The beetles, commonly referred to as the fir engraver (*Scolytus ventralis* Lec.) and the Douglas fir engraver (*Scolytus unispinosus* Lec.) are described by KEEN (1952) with respect to their life cycle and habits. *Endomycopsis scolyti* was found almost free of other yeasts in both species of bark beetles and in insect frass occurring in both species of trees in widely separated areas of California. In view of the highly specific association of this yeast with the above-named bark beetles, the specific epithet of the yeast was derived from the genus name of the beetles.

Standard description of *Endomycopsis scolyti* nov. spec.

Growth in malt extract: After 3 days at room temperature the cells of one mating type (culture 59-62, type a) are almost round, short oval or ellipsoidal (1.8 — 2.3) × (2.3 — 3.9) μ. Cells of the other mating type (culture 59-136, type b) are slightly larger (1.8 — 3.2) × (3.2 — 5.8) μ, occasionally reaching a length of 7.3 μ. Cells of both types occur singly, in pairs, or in short chains. A slight ring and sediment are formed. After one month a thin ring and sediment, but no pellicle.

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Streak culture on malt agar: After 1 month the culture is cream colored to white; smooth with slight transverse folds, glistening; texture pasty; cross section is convex in the center with a flat spreading periphery; border shows extensive pseudomycelial or mycelial growth.

Slide culture on potato glucose agar: Extensive pseudomycelium of the Mycocandida type as well as true hyphae.

Sporulation: The yeast is heterothallic and conjugation and sporulation occur only upon mixing of the appropriate mating types. Asci contain one to four ascospores. Spores globose to saturn-shaped (diameter 2.2 μ) or indistinctly hat-shaped with a short brim (1.8—2.2 μ). They contain a small refractile body. Asci rupture soon after formation. Conjugation tube and zygote formation occurred readily, but few ascospores were produced.

Fermentation: glucose + (slow); galactose + (latent, weak); sucrose (latent, very weak); maltose (latent, very weak); lactose —; raffinose — (occasionally a few gas bubbles); melibiose —.

Assimilation: Positive: glucose, galactose, maltose, sucrose, cellobiose, trehalose, lactose (weak), melibiose, raffinose, melezitose, D-xylose, L-arabinose, D-arabinose (latent, weak), D-ribose (latent), L-rhamnose, ethanol, glycerol, i-erythritol, adonitol, D-mannitol, D-sorbitol, α-methyl-D-glucoside (variable), salicin, glucono-δ-lactone (weak), succinic acid (latent) and citric acid (variable).

Negative: L-sorbose, inulin, soluble starch, dulcitol, Ca-2-keto-gluconate, K-5-keto-gluconate, DL-lactic acid, i-inositol.

Assimilation of nitrate or nitrite: Negative.

Growth in vitamin-free mineral medium: Negative (both mating types require biotin and thiamine).

Growth at 37° C.: Positive.

Type strains of the two mating types:

59-62 isolated from frass of *Scolytus ventralis* in *Abies concolor* at Sly Park Lake, El Dorado County, California.

59-136 isolated from frass of *Scolytus ventralis* in *Abies concolor* at Swain Mountain, Lassen County, California.

The areas of isolation are about 120 miles apart and both are located in the so-called "Yellow Pine Forest" as defined by Munz and Keck (1959).

E. scolyti is a weakly fermentative yeast. In Durham tubes the first visible formation of gas occurs after about 5 days with glucose. With galactose it takes 7 days, and with sucrose and maltose 12-15 days. Full vials of gas are usually obtained with glucose and galactose, but with sucrose and maltose rarely more than half of the volume of the vials becomes filled with carbon dioxide.

The two mating types occurred approximately with equal frequency among the many strains isolated and, moreover, occurred side by side in brood material obtained from the same tree. The sexual vigor, expressed by the rapidity of conjugation tube and zygote formation (as well as the percentage of vegetative cells participating in this process) varied considerably among the strains. Although the two selected mating types behaved satisfactorily with respect to zygote formation, the number of zygotes producing ascospores was always exceedingly small. The best sporulation medium found had the following composition: ground sprouted barley 10 g (wet basis), *Scolytus* frass from fir 3 g, agar 2.5 g, distilled water to make 100 ml. The pH was adjusted to 5.5. The barley had been sprouted for 5 days under commercial conditions in a local malt house and was stored in frozen condition in our laboratory.

With the idea that the process of conjugation *versus* meiosis and spore formation might be favored by somewhat different optimal conditions, an attempt was made to produce a diploid culture of the yeast. It would then be possible to study the optimal conditions of sporulation, without having to consider zygote formation. Professor ROBERT K. MORTIMER of the University of California at Berkeley was kind enough to supply the authors with several nutritionally deficient mutants of each mating type, which were produced by ultraviolet irradiation. An adenine dependent (pinkish) mutant of one mating type was mixed on sporulation medium with an arginine dependent mutant of the other mating type. After about 7 days, when zygotes were detected, the culture was streaked on a glucose containing minimal medium (a special type of yeast nitrogen base, Difco, devoid of amino acids). Only zygotes should be able to grow on this medium. As a control, to check for back mutation, each mating type was also plated in comparable concentration on minimal medium. A few white colonies appeared when the sporulating culture was streaked on minimal medium, whereas the individual mating types produced no growth. After two successive replatings on minimal agar (to remove haploid cells) the diploid culture was placed on sporulation medium and on wort agar. On both media excellent

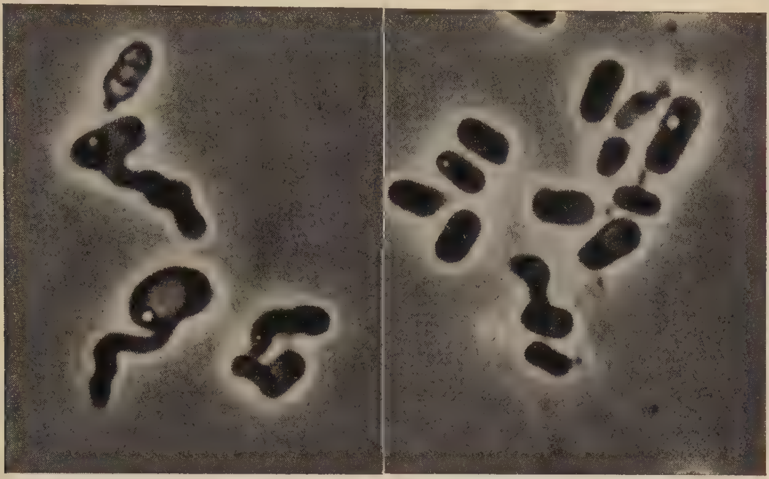

Fig. 1. Left: Mixture of the normal mating types of *E. scolyti* forming conjugation tubes. Right: ditto, showing a zygote (phase contrast microscopy).

sporulation occurred. Fig. 1 shows conjugation tubes and a zygote produced by the normal mating types. Fig. 2 shows an ascus and spores in the process of liberation from the ascus. This series represents cells formed from the two mutant mating types and the asci appear to be diploid cells.

During the isolation of the yeast it was noted that *E. scolyti* may occur naturally as a smooth and as a rough form, the giant colonies

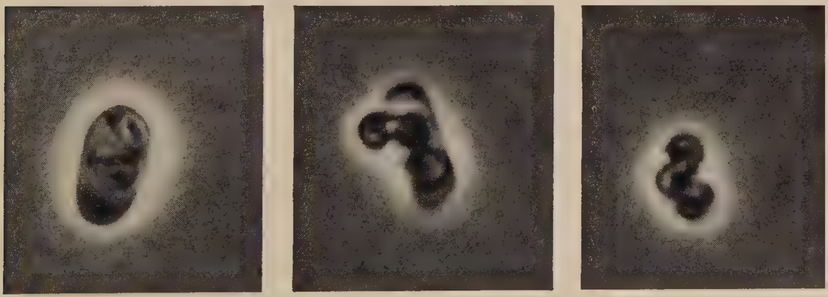

Fig. 2. Various stages during sporulation of a diploid culture of *E. scolyti* (obtained by mixing mutant mating types). Left: Ascus containing two hat-shaped ascospores; center: three ascospores in the process of liberation from the ascus; right: group of three liberated ascospores (phase contrast microscopy).

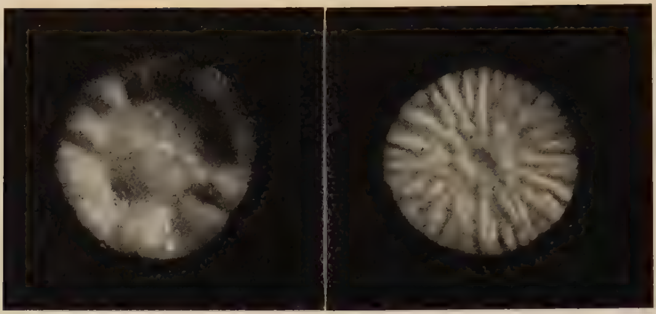

Fig. 3. Giant colonies on wort gelatin after 4 days at 18° C. Left: normal smooth strain of *E. scolyti*. Right: same of a rough strain of *E. scolyti*.

of which are shown in Fig. 3. The above discussion and description are based on the smooth type. The rough type differs in colony morphology, and in a weaker mating reaction (rough × smooth and rough × rough). Physiologically it differs in assimilating lactose, glucono-δ-lactone and succinic acid more strongly, whereas α-methyl-D-glucoside was always positive rather than being variable. Colonies produced from *Scolytus ventralis* material were predominantly of the smooth type, whereas colonies arising from *S. unispinosis* material showed a majority of the wrinkled or rough type.

Discussion.

Generic classification of the new yeast posed some difficulties. The organism might fit in the genus *Pichia* as amended by Phaff (1956), were it not for its heterothallic nature and for the formation, under certain conditions, of true hyphae. Members of the genus *Pichia* may be haploid or diploid but all are homothallic. Although the present definition of the genus *Endomycopsis* is not quite adequate to accommodate a species such as the one described above, both Wickerham and Burton (1954) and van der Walt (1959) have placed provisionally a number of species in this genus which have several properties in common with our new species. All are heterothallic, formation of true mycelium is limited and several are associated with insects which attack coniferous or broad-leaved trees. The yeasts referred to are the perfect form of *Candida guilliermondii* (Cast.) Langeron et Guerra, *Candida membranaefaciens* Wickerham et Burton and *Endomycopsis wickerhamii* van der Walt. *Endomycopsis ohmeri* Etchells et Bell may also be included in this category. The last species was first described as a homothallic yeast (ETCHELLS and BELL, 1950), but was later shown to be heterothallic by WICKERHAM and BURTON (1952). Later, WICKERHAM and BURTON (1954) demonstrated that *Candida guilliermondii* var. *membranaefaciens* and *Candida chalmersi* are imperfect forms of *E. ohmeri*.

The authors also wish to place the new species provisionally in the genus *Endomycopsis* among the, perhaps primitive, group of heterothallic insect — tree dependent species. Future research is needed to establish whether this group should ultimately remain in *Endomycopsis*, be included in *Pichia* or in a more sharply defined genus *Petasospora* (VAN DER WALT, 1959), or form an independent genus.

Latin diagnosis of *Endomycopsis scolyti* nov. spec.

In extracto malti cellulae rotundae aut ovoidae, (1.8 — 2.3) × (2.3 — 3.9) μ, forma a; aut (1.8 — 3.2) × (3.2 — 5.2 — 7.3) μ, forma b; singulae, binae aut catenatae; sedimentum et annulus formantur. In agaro maltato cultura albida, glabra, nitida, mollis, margine piloso. Pseudomycelium et mycelium verum. Oriuntur asci ex conjugatione inter cellulas sexus contrarii haploideas. Ascosporae subovoidae aut pileiformes, 1—4 in quoque ascus. Ascosporae ex ascus celeriter liberantur. Fermentatio glucosi (leniter), galactosi (lente), sacchari (exigui), maltosi (exigui). In medio minerali cum glucoso, galactoso, maltoso, saccharoso, cellobioso, trehaloso, lactoso (exiguo), melibioso, raffinoso, D-xyloso, L-arabinoso, D-arabinoso (lento, exiguo), D-riboso (lento), L-rhamnoso, alcohole aethylico, glycerole, i-erythrytole, adonitole, D-mannitole, D-sorbitole, alpha-methylglucoside (variabile), salicine, acidum gluconum (exiguo) et acidum succinum (lente), crescit. Nitras kali non assimilatur. Necessariae ad crescentiam sunt vitaminae externae. Habitatio in *Scolytus ventralis* et *Scolytus unispinosus* in *Abies* et *Pseudotsuga*.

Summary.

A description has been given of a new heterothallic species of yeast, *Endomycopsis scolyti*. The natural habitat of the new species is in the frass or in larvae or adults of *Scolytus ventralis* and *Scolytus unispinosus*, two species of bark beetles which attack *Abies* and *Pseudotsuga*, respectively.

Acknowledgement.

The authors express their appreciation to Professor ROBERT K. MORTIMER for supplying the nutritionally deficient mutants of the mating types and to Mr. H. TANAKA for assistance in preparing the photomicrographs. The authors are also grateful to the staff of the United States Department of Agriculture, Forest Insect Investigations, Berkeley, California for supplying brood material and for the identification of the bark beetles.

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(From the National Institute of Public Health, Utrecht, the Netherlands).

FOUR NEW *SALMONELLA* TYPES (*S. GAMBAGA, S. KUMASI, S. PRAMISO* AND *S. ASHANTI*) ISOLATED IN GHANA

by

P. A. M. GUINÉE and **E. H. KAMPELMACHER**

(Received November 28, 1960).

All four *Salmonella* types to be described were isolated by D. Zwart, Kumasi, College of Technology, Ghana.

S.ashanti was isolated from a rat, the other three types from reptiles. All the strains showed the typical biochemical behaviour of the genus *Salmonella*. In table 1 the biochemical data are shown.

In table 2 and 3 the serological data are given for the four new *Salmonella* types.

These data lead to the antigenic formulas for the four new types:

S.gambaga	21; z35 : enz15
S.kumasi	30; z10 : enz15
S.pramiso	3,10; c : 1,7
S.ashanti	28; b : 1,6

We are indebted to Prof. Dr. F. Kauffmann for confirmation of our findings.

Summary.

Four new *Salmonella* types isolated in Ghana are described. *S.gambaga*, 21; z35 : enz15, *S.kumasi*, 30; z10 : enz15, and *S.pramiso*, 3,10; c : 1,7, were isolated from reptiles; *S.ashanti*, 28; b : 1,6, was isolated from a rat.

T

Biochemical behaviour of *S. ga*

	glucose + gas	mannitol	adonitol	dulcitol	inositol	lactose	salicine	sucrose	indol	T.S.I.	ammoniumcitrate	methylred
S. gambaga	++	+	—	+	—	—	—	—	—	+	+	+
S. kumasi	++	+	—	+	—	—	—	—	—	+	+	+
S. pramiso	++	+	—	+	+	—	—	—	—	+	+	+
S. ashanti	++	+	—	+	—	—	—	—	—	+	+	+

TABLE 2.

Control of O-antigens of *S.gambaga*, *S. kumasi*, *S. pramiso* and *S. ashanti*.

serum	antigens	
	S. gambaga	*S. minnesota*
S. gambaga	1 : 1280	1 : 1280
S. gambaga absorbed with *S. minnesota*	—	—
	S. kumasi	*S. urbana*
S. kumasi	1 : 2500	1 : 2500
S. kumasi absorbed with *S. urbana*	—	1 : 20
	S. pramiso	*S. anatum*
S. pramiso	1 : 1280	> 1 : 5000
S. pramiso absorbed with *S. anatum*	1 : 20	1 : 80
	S. ashanti	*S. tel-aviv*
S. ashanti	1 : 1280	1 : 640
S. ashanti absorbed with *S. tel-aviv*	1 : 40	—

i, S. pramiso and *S. ashanti.*

phenyl-alanine	malonate	lysine decarboxylase	KCN	arabinose	rhamnose	trehalose	xylose	glycerol (Stern)	d-tartrate	l-tartrate	i-tartrate	citrate	mucate	maltose	sorbitol	gelatin
—	—	+	—	+	+	+	+	+	+	+	—	+	+	+	+	—
—	+	+	—	+	+	+	—	+	+	+	—	+	+	+	+	—
—	—	+	—	+	+	+	—	+	+	+	+	+	+	+	+	—
—	—	+	—	+	+	+	+	+	+	+	—	+	+	+	+	—

TABLE 3.

Control of H-antigens of *S. gambaga, S. kumasi, S. pramiso* and *S. ashanti.*

serum	antigens		
	S. gambaga	*S. chittagong*	*S. durban*
S. gambaga	> 1 : 1280	> 1 : 1280	1 : 640
S. gambaga absorbed with *S. chittagong*	1 : 640	—	1 : 160
S. gambaga absorbed with *S. durban*	1 : 320	1 : 1280	—
	S. kumasi	*S. guinea*	*S. durban*
S. kumasi	> 1 : 1280	> 1 : 1280	> 1 : 1280
S. kumasi absorbed with *S. guinea*	1 : 1280	—	1 : 640
S. kumasi absorbed with *S. durban*	1 : 1280	1 : 320	—
	S. pramiso	*S. altendorf*	*S. bredeney*
S. pramiso	> 1 : 1280	> 1 : 1280	> 1 : 1280
S. pramiso absorbed with *S. altendorf*	1 : 160	1 : 40	(1)
S. pramiso absorbed with *S. bredeney*	> 1 : 1280	(1)	—
	S. ashanti	*S. java*	*S. anatum*
S. ashanti	> 1 : 1280	> 1 : 1280	> 1 : 1280
S. ashanti absorbed with *S. java*	1 : 1280	—	(1)
S. ashanti absorbed with *S. anatum*	> 1 : 1280	(1)	—

(1) not done

(National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa).

THREE NEW ANASCOSPOROGENOUS YEASTS

by

J. P. VAN DER WALT and **AMELIA E. VAN KERKEN**

(Received January 4, 1961).

Three new asporogenous yeast species have been recovered from materials associated with wine making. The following descriptions of these species, *Torulopsis cantarellii*, *Torulopsis vanzylii* and *Torulopsis capsuligenus*, are based on the standard methods and procedures of WICKERHAM (1951) and LODDER and KREGER-VAN RIJ (1952).

Torulopsis cantarellii nov. spec.

DESCRIPTION.

Growth in malt extract: After 3 days at 25° C., the cells are round to short oval, (2.4 — 5.4) × (3.0 — 6.0) μ, single, in pairs or small chains. After 1 month at 17° C., a sediment is formed and occasionally, a ring.

Growth on malt agar: After 3 days at 25° C., the cells are round, short oval to oval, (2.4 — 6.0) × (3.0 — 7.7) μ, single or in pairs. After 1 month at 17° C., the streak culture is butyrous, cream-coloured, usually with some faint structure and somewhat shiny. The margin is smooth or, occasionally, with some faint structure.

Slide cultures: No pseudomycelium is formed.

Growth at 37° C.: Absent.

Fermentation:

Glucose	+	Maltose	—
Galactose	—	Lactose	—
Saccharose	—	Raffinose	—
Trehalose	+	(slow)	

Carbon assimilation: Glucose, galactose (latent), L-sorbose, trehalose, D-ribose (latent), D-mannitol, D-sorbitol, adonitol, i-erythritol, glycerol, ethanol, potassium D-gluconate (variable), succinic acid and DL-lactic acid (variable) are assimilated. Saccharose, maltose, cellobiose, melibiose, lactose, raffinose, melizitose, D-arabinose, L-arabinose, L-xylose, L-rhamnose, salicin, α-methyl-D-glucoside, i-inositol, dulcitol and citric acid are not assimilated.

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Absent.

Growth in the absence of an extraneous vitamin source: Positive.

DISCUSSION.

Ten strains of the species were isolated from industrial grape musts treated with actidione and ethanol (*cf.* VAN DER WALT and VAN KERKEN, 1961).

The ten strains were mated according to the methods of WICKERHAM and BURTON (1954). Sporulation was, however, not observed.

In view of the apparent absence of ascospore formation and the inability to form pseudomycelium, the species is assigned to the genus *Torulopsis* (*in sensu* LODDER et KREGER-VAN RIJ).

Since the species cannot be identified with any member of the genus hitherto described, the ten strains are considered representative of a new species. For this new species, the name *Torulopsis cantarellii* is proposed in honour of Dr. CORRADO CANTARELLI, being in recognition of his extensive studies on the yeast flora of the vinous fermentation.

Torulopsis vanzylii nov. spec.

DESCRIPTION

Growth in malt extract: After 3 days at 25° C., the cells are round to short oval, (2.4 — 6.0) × (2.7 — 6.6) μ, single or in pairs. After 1 month at 17° C., a sediment and ring are formed.

Growth on malt agar: After 3 days at 25° C., the cells are short oval to oval, (2.0 — 4.8) × (2.4 — 5.4) μ, single or in pairs. After 1 month at 17° C., the streak culture is butyrous, greyish-white, somewhat shiny, flat and smooth. The margin is smooth or slightly undulating.

Slide cultures: A few chains or branched chains of cells are formed. No characteristic pseudomycelium is formed.

Growth at 37° C.: Absent.

Fermentation:

Glucose	+ (weak and slow)	Maltose	—
Galactose	—	Lactose	—
Saccharose	—	Raffinose	—

Carbon assimilation: Glucose, cellobiose, L-xylose, L-rhamnose, salicin, D-mannitol, D-sorbitol, glycerol, ethanol, citric acid, succinic acid and DL-lactic acid are assimilated. Galactose, L-sorbose, saccharose, maltose, trehalose, melibiose, lactose, raffinose, melizitose, D-arabinose, L-arabinose, D-ribose, α-methyl-D-glucoside, i-inositol, dulcitol, adonitol, i-erythritol and potassium D-gluconate are not assimilated.

Assimilation of nitrate and nitrite: Positive.

Splitting of arbutin: Positive.

Growth in the absence of an extraneous vitamin source: Absent.

DISCUSSION.

The culture was received from Dr. J. A. VAN ZYL who isolated this yeast from the mould-growth developing on a floor of a refrigerated wine cellar.

As only one strain was encountered, it was not possible to establish whether this isolate represents a haploid heterothallic mating type of some nitrate-positive ascosporogenous yeast species.

Since the strain is apparently asporogenous, fermentative, albeit weakly, and forms no pseudomycelium, it is assigned to the genus *Torulopsis* (*in sensu* LODDER et KREGER-VAN RIJ). As it cannot be identified with any of the hitherto described members of the genus, the strain is considered representative of a new species.

For this new species the name *Torulopsis vanzylii* is proposed in honour of Dr. J. A. VAN ZYL who first isolated the species.

Torulopsis capsuligenus nov. spec.

DESCRIPTION.

Growth in malt extract: After 3 days at 25° C., the cells are short oval to long oval, single or in pairs and encapsulated. The size of the cells measured without the capsule is (3.0 — 6.6) × (3.7 — 10.8) μ. A ring and sediment are formed. After 7 days a thin pellicle is formed. This pellicle may drop to the bottom so that after 1 month only a sediment, islets and a ring are formed.

Growth on malt agar: After 3 days at 25° C., the cells are short oval to long oval, single or in pairs and encapsulated. The size of the cells measured without the capsule is (3.0 — 6.6) × (3.6 — 8.7) μ. After 1 month at 17° C., the streak culture is butyrous to almost mucoid, yellowish-brown, strongly glistening, somewhat raised, and occasionally with some structure. The margin is undulating.

Slide cultures: No pseudomycelium is formed.

Growth at 37° C.: Absent.

Formation of starch-like compounds: Positive.

Fermentation:

Glucose	+ (weak and slow)	Maltose	—
Galactose	—	Lactose	—
Saccharose	—	Raffinose	—

Carbon assimilation: Glucose, galactose, maltose, trehalose, cellobiose, D-arabinose, L-arabinose, L-xylose, salicin, α-methyl-D-glucoside, D-mannitol, D-sorbitol, i-inositol, dulcitol, adonitol, glycerol, ethanol, potassium D-gluconate, citric acid, and succinic acid are assimilated. L-sorbose, saccharose, melibiose, lactose, raffinose, melizitose, L-rhamnose, D-ribose, i-erythritol, and DL-lactic acid are not assimilated.

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Positive (weak).

Growth in the absence of an extraneous vitamin source: Positive.

Discussion.

One strain of the above described species was received from Dr. J. A. van Zyl who obtained the culture from the laboratories of the "Ko-operatieve Wijnbouwers Vereniging van Zuid-Afrika". Further details are not available.

With the present status of the taxonomy of the anascosporogenous yeasts, the exact generic position of the strain is uncertain.

As the strain is apparently anascosporogenous, forms no pseudomycelium and is fermentative, albeit weakly, its inclusion in the genus *Torulopsis* (*in sensu* LODDER et KREGER-VAN RIJ) seems acceptable. On the other hand, the strain produces starch-like compounds and encapsulated cells which are properties typical of the genus *Cryptococcus* (*in sensu* LODDER et KREGER-VAN RIJ).

However, since the genus *Cryptococcus* is strictly oxidative, and as starch production has already been observed in other anascosporogenous genera, *e.g. Candida, Trichosporon, Rhodotorula* and *Bullera* (LODDER, SLOOFF and KREGER-VAN RIJ, 1958), the authors favour provisionally assigning the strain to the genus *Torulopsis*. The strain cannot be identified with any of the hitherto described species. It is considered therefore to be representative of a new species.

For this new species the name *Torulopsis capsuligenus* is proposed.

Torulopsis cantarellii nov. spec.

In extracto malti cellulae rotundae aut subovoidiae (2.4 — 5.4) × (3.0 — 6.0) μ, singulae aut binae. Sedimentum et aliquando annulus formantur.

In agar malti, cellulae rotundae, subovoidiae aut ovidiae (2.4 — 6.0) × (3.0 — 7.7) μ, singulae aut binae. Cultura (post unum mensem 17° C.) butyrosa, albiflava, plerumque cum structura parva, parum nitens. Margine glabro, plerumque cum structura parva.

Pseudomycelium nullum.

Crescentia fieri non potest in 37° C.

Glucosum et trehalosum (lente) fermentantur at non galactosum, saccharosum, maltosum, lactosum nec raffinosum.

Glucosum, galactosum (lente), L-sorbosum, trehalosum, D-ribosum (lente), D-mannitolum, D-sorbitolum, adonitolum, i-erythritolum, glycerolum, ethanolum, kalium D-gluconicum (var.), acidum succinicum et DL-acidum lacticum (var.) assimilantur, at non saccharosum, maltosum, cellobiosum, melibiosum, lactosum, raffinosum, melizitosum, D-arabinosum, L-arabinosum, L-xylosum, L-rhamnosum, salicinum, α-methyl-D-glucosidum, i-inositolum, dulcitolum nec acidum citricum.

Kalium nitricum et natrium nitrosum non assimilantur.

Arbutinum non finditur.

Ad crescentiam vitaminae externae non necessariae sunt.

Torulopsis vanzylii nov. spec.

In extracto malti, cellulae rotundae aut subovoidiae (2.4 — 6.0) × (2.7 — 6.6) μ, singulae aut binae. Sedimentum et annulus formantur.

In agar malti cellulae subovoidiae aut ovoidiae (2.0 — 4.8) × (2.4 — 5.4) μ, singulae aut binae. Cultura (post unum mensem 17° C.) butyrosa, griseola-alba, plana, glabra, nitida. Margine glabro aut paulum undulato.

Pseudomycelium nullum; interdum blastosporae in catenis parvis aut racemis parvis.

Crescentia fieri non potest in 37° C.

Glucosum tardum et exiguum fermentatur at non galactosum, saccharosum, maltosum, lactosum nec raffinosum.

Glucosum, cellobiosum, L-xylosum, L-rhamnosum, salicinum, D-mannitolum, D-sorbitolum, glycerolum, ethanolum, acidum citricum, acidum succinicum et DL-acidum lacticum assimilantur at non galactosum, L-sorbosum, saccharosum, maltosum, trehalosum, melibiosum, lactosum, raffinosum, melizitosum, D-arabinosum, L-arabinosum, D-ribosum, α-methyl-D-glucosidum, i-inositolum, dulcitolum, adonitolum, i-erythritolum nec kalium D-gluconicum.

Kalium nitricum et natrium nitrosum assimilantur.

Arbutinum finditur.

Necessariae ad crescentiam sunt vitaminae externae.

Torulopsis capsuligenus nov. spec.

In extracto malti cellulae subovoidiae longovoidiaeque, singulae aut binae, capsulae circumdatae. Sine capsulis dimensiones cellularum sunt (3.0 — 6.6) × (3.7 — 10.8) μ. Sedimentum, annulus et pellicula tenuis aut insulae formantur. Pellicula facile cadet ad fundum.

In agar malti cellulae subovoidiae, longovoidiaeque, singulae aut binae, capsulae circumdatae. Sine capsulis dimensiones cellularum sunt (3.0 — 6.6) × (3.6 — 8.7) μ. Cultura (post unum mensem 17° C.) butyrosa paene mucosa, nitidissima, parum acclivis, aliquando cum structura parva, flavifusca. Margine undulato.

Pseudomycelium nullum.

Amylosum formatur.

Crescentia fieri non potest in 37° C.

Glucosum tardum et exiguum fermentatur at non galactosum, saccharosum, maltosum, lactosum nec raffinosum.

Glucosum, galactosum, maltosum, trehalosum, cellobiosum, D-arabinosum, L-arabinosum, L-xylosum, salicinum, α-methyl-D-glucosidum, D-mannitolum, D-sorbitolum, i-inositolum, dulcitolum, adonitolum, glycerolum, ethanolum, kalium D-gluconicum, acidum citricum et acidum succinicum assimilantur at non L-sorbosum, saccharosum, melibiosum, lactosum, raffinosum, melizitosum, L-rhamnosum, D-ribosum, i-erythritolum nec DL-acidum lacticum.

Kalium nitricum et natrium nitrosum non assimilantur.

Arbutinum finditur (paulum).

Vitaminae externae ad crescentiam non necessariae sunt.

Cultures of *Torulopsis cantarellii*, *Torulopsis vanzylii* and *Torulopsis capsuligenus* have been deposited in the yeast collection of the Centraalbureau voor Schimmelcultures in Delft.

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THE SYNERGISM BETWEEN PENICILLIN AND KANAMYCIN

by

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In the past few years the antibiotic kanamycin has rapidly taken its place in the series of routinely used antibiotics. Since kanamycin was first mentioned by UMEZAWA (10), most of the characteristics of this antibiotic have been submitted to exhaustive investigation within a few years. The various aspects of kanamycin were elucidated in detail in the Annals of the New York Academy of Sciences (1958, vol 76), among other publications. Kanamycin has proved to be a chiefly bactericidal broad-spectrum antibiotic, closely related to neomycin, with which cross resistance exists (6). Its minimum bactericidal concentration is twice as high, on average, as its minimum bacteriostatic concentration. In addition to yielding good clinical results in infections with *Staphylococcus aureus*, *Proteus*, *Salmonella* and *E. coli*, kanamycin proved to be effective against *M. tuberculosis*, both in vivo (9,13) and in vitro (2,9). Kanamycin is not free of toxic effects, chiefly involving the kidneys (3, 7, 12) and the 8th cranial nerve (4, 5, 7, 12).

The efficacy of kanamycin combined with other antibiotics has likewise been studied by a number of investigators. GOUREVITCH *et al.* (6) observed that, in a culture of *Staphylococcus aureus*, the combination of kanamycin and penicillin killed a larger percentage of bacteria than did each of the antibiotics separately; these authors formed no conclusion as to synergism on the basis of this observation. ANDRIEU *et al.* (1) carried out in vitro experiments, after which they concluded in favour of a synergism between kanamycin and penicillin against both penicillin-susceptible and penicillin-resistant strains of *Staphylococcus aureus* and against *Proteus*, some strains of *E. coli* and *Enterococcus*. JADIN (8) and VAN DER VOORDE (11) found that the minimum bactericidal concentration of kanamycin against *Staphylococcus* was reduced to about 1/8 in the presence of low penicillin concentrations.

Although a synergism found in vitro between two antibiotics does not necessarily have to occur in vivo, yet in vitro determinations must constitute the indication for in vivo application of a combination of antibiotics, as long as no simple methods are available by which synergism can be demonstrated in vivo. The results of investigations made by JADIN (8) suggest that the use of a combination of kanamycin and penicillin opens perspectives in practice, not in the least because of the possibility, in some cases, to obtain results with a low dosage of kanamycin, with the associated reduced risk of toxic reactions.

By in vitro testing of the efficacy of the combination of penicillin and kanamycin against a number of microorganisms, we attempted to collect data suggestive of the area in which indications for combined use of the two antibiotics might be found. Interpretation of the findings obtained, however, must be exclusively dependent for the time being on the minimum inhibitory concentrations of the antibiotics in relation to various microorganisms, and on the attainable serum and urinary concentrations of these antibiotics.

METHODS.

The bacterial strains used in our experiments have all been isolated from material sent to the laboratory to be tested for pathogenic microorganisms. The majority of the strains of *Staphylococcus aureus* were isolated from pus, and the remaining strains for the most part originated from infected urine.

Determinations of minimum bacteriostatic and bactericidal concentrations of kanamycin¹), penicillin and the two antibiotics in combination, were made by the tube dilution method. Into 1 ml of an antibiotic dilution in meat broth, 1 drop (1/30 ml) of a 1 : 20 dilution of a fresh 24-hour culture of the microorganism to be tested in meat broth was inoculated. The lowest antibiotic concentration, which prevented bacterial growth after incubation for 18-20 hours

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¹) We are indebted to Continental Pharma for supplying the kanamycin.

was recorded as minimum bacteriostatic concentration. By inoculation on bloodagar from the tubes in which no growth had occurred, the minimum bactericidal concentration was determined (less than 10 colonies after inoculation on agar).

Virtually all determinations were made twice. Only in a few strains of *Staphylococcus aureus* were differences in susceptibility to penicillin and to the combination observed between two determinations. The cause is probably to be found in a varying penicillinase production. In these cases the results of determinations simultaneously made in one strain are reported. Only those concentrations of the combined antibiotics were investigated that can be obtained at a normal and sometimes at a high (penicillin) dosage in serum or urine.

The term synergism signifies that the joint effect of two antibiotics exceeds the sum of the effects of the two antibiotics separately. We may assume a synergistic bacteriostatic effect (Tables 1—5) if the following ratios exceed 2:

a. The ratio between the minimum bacteriostatic concentration of kanamycin alone (MCS K) and that of kanamycin combined with penicillin (MCS K+P).

b. The ratio between the minimum bacteriostatic concentration of penicillin alone (MCS P) and that of penicillin combined with kanamycin (MCS P+K).

We may assume a synergistic bactericidal effect (Tables 1—5) if the following ratios exceed 2:

c. The ratio between the minimum bactericidal concentration of kanamycin alone (MCC K) and that of kanamycin combined with penicillin (MCC K+P).

d. The ratio between the minimum bactericidal concentration of penicillin alone (MCC P) and that of penicillin combined with kanamycin (MCC P+K).

RESULTS.

a. Penicillin-sensitive *Staphylococcus aureus* (Table 1).

In these strains there is certainly a synergism between penicillin and kanamycin. This is especially clearly manifested in the minimum bactericidal concentrations.

b. Penicillin-resistant *Staphylococcus aureus* (Table 2).

In the case of these strains, the minimum bactericidal concentration of kanamycin can be lowered by combining kanamycin with penicillin (usually in high concentrations). The average minimum bactericidal concentration of kanamycin in combination with penicillin is 1/4 of that of kanamycin alone. Only in strain no. 2 a synergistic bactericidal effect was not demonstrable.

c. *Enterococci* (Table 3).

Of the three enterococcal strains included in the investigation there were two (no. 25 and no. 26) which showed no synergism; the third (no. 40) did show a synergism. In the case of this strain, however, high concentrations of the two antibiotics in combination were required to attain a bactericidal effect.

d. *Klebsiella* (Table 3).

The two *Klebsiella* strains investigated differed in behaviour. In one (no. 23), the minimum bacteriostatic and bactericidal concentrations of kanamycin combined with penicillin were 1/8 and 1/4, respectively, of those of kanamycin alone; in the case of the other strain (no. 24), no synergism can be assumed.

e. *Escherichia coli* (Table 4).

In two of the three strains (no. 21 and no. 22) there was certainly a synergism between kanamycin and penicillin, although relatively high concentrations of penicillin were required for this (these are attainable in urine). In the third strain of *E. coli* (no. 33), only a synergistic bacteriostatic effect was demonstrable. *E. coli*, therefore, also showed some difference of behaviour relative to the two antibiotics in combination.

f. *Pseudomas aeruginosa* (Table 4).

In one strain (no. 27), synergism between penicillin and kanamycin was observed; this was not definitely established in the other strain (no. 28). It is doubtful whether the high concentrations of antibiotics at which synergism is found, can be expected in vivo, even in the urine.

g. *Proteus* (Table 5).

In all *Proteus* strains included in the investigation, an unmistakable synergism between the two antibiotics was established, both in bacteriostatic and bactericidal effect. The kanamycin concentrations required in the combination to obtain a bactericidal effect can be attained in the urine even at a low dosage.

TABLE 1.

Minimum bacteriostatic (MCS) and bactericidal (MCC) concentrations of penicillin (P) in U/ml, kanamycin (K) in γ/ml and of the combination of both in penicillin-sensitive strains of *Staphylococcus aureus*. Ratios between these concentrations of the separate antibiotics and those of the antibiotics in the combination.

Strain	P		K		P + K	P + K	MCS P	MCC P	MCS K	MCC K
	MCS	MCC	MCS	MCC	MCS	MCC	MCS P+K	MCC P+K	MCS K+P	MCC K+P
no. 3	0.05	0.1	6.25	25	0.02 + 1.56	0.02 + 1.56	2.5	5	4	16
no. 9	0.05	0.1	6.25	25	0.02 + 1.56	0.02 + 1.56	2.5	5	4	16
no. 10	0.05	0.05	6.25	12.5	0.02 + 3.12	0.02 + 3.12	2.5	2.5	2	4
no. 11	0.05	0.1	6.25	12.5	0.02 + 1.56	0.02 + 1.56	2.5	5	4	8

TABLE 2.

Minimum bacteriostatic (MCS) and bactericidal (MCC) concentrations of penicillin (P) in U/ml, kanamycin (K) in γ/ml and of the combination of both in penicillin-resistant strains of *Staphylococcus aureus*. Ratios between these concentrations of the separate antibiotics and those of the antibiotics in the combination.

Strain	P		K		P + K	P + K	MCS P	MCC P	MCS K	MCC K
	MCS	MCC	MCS	MCC	MCS	MCC	MCS P+K	MCC P+K	MCS K+P	MCC K+P
no. 2	50	400	3.12	3.12	0.5 + 0.78	0.5 + 3.12	100	400	2	1
					2 + 0.78	2 + 1.56	25	200	4	2
					5 + 0.39	5 + 1.56	10	80	8	2
no. 5	50	100	3.12	6.25	0.5 + 3.12	0.5 + 6.25	100	200	1	1
					1 + 1.56	1 + 3.12	50	100	2	2
					5 + 0.78	5 + 1.56	10	20	4	4
no. 6	100	400	3.12	6.25	1 + 0.78	1 + 1.56	100	400	4	4
no. 7	50	100	3.12	12.5	0.5 + 1.56	2 + 3.12	100	50	2	4
					5 + 0.78	5 + 3.12	10	20	4	4
no. 15	100	400	3.12	12.5	1 + 0.78	1 + 1.56	100	400	4	8

TABLE 3.

Minimum bacteriostatic (MCS) and bactericidal (MCC) concentrations of penicillin (P) in U/ml, kanamycin (K) in γ/ml and of the combination of both in various bacterial strains. Ratios between these concentrations of the separate antibiotics and those of the antibiotics in the combination.

Strain	P		K		P + K	P + K	MCS P	MCC P	MCS K	MCC K
	MCS	MCC	MCS	MCC	MCS	MCC	MCS P+K	MCC P+K	MCS K+P	MCC K+P
no. 25 *Enterococcus*	5	5	50	50	2 + 6.25	—	2.5	—	8	—
no. 26 *Enterococcus*	2	5	50	50	2 + 6.25	—	1	—	8	—
no. 40 *Enterococcus*	10	20	50	100	1 +25 5 + 3.12	10 + 1.56 5 + 12.5 2 + 25	10 2	2 16 10	2 4	64 8 4
no. 23 *Klebsiella*	>6400		25	25	50 + 3.12	50 + 6.25	∞	∞	8	4
no. 24 *Klebsiella*	400	400	6.25	6.25	1 + 3.12	1 + 6.25	400	400	2	1

TABLE 4.

Minimum bacteriostatic (MCS) and bactericidal (MCC) concentrations of penicillin (P) in U/ml, kanamycin (K) in γ/ml and of the combination of both in various bacterial strains. Ratios between these concentrations of the separate antibiotics and those of the antibiotics in the combination.

Strain	P		K		P + K	P + K	MCS P	MCC P	MCS K	MCC K
	MCS	MCC	MCS	MCC	MCS	MCC	MCS P+K	MCC P+K	MCS K+P	MCC K+P
no. 21 *E. coli*	100	400	25	50	5 +12.5 10 + 6.25	5 + 25 20 + 6.25 50 + 3.12	20 10	80 20 8	2 4	2 8 16
no. 22 *E. coli*	50	50	25	25	2 +12.5	5 + 6.25 10 + 1.56	25	10 5	2	4 16
no. 33 *E.coli*	100	200	25	25	0.5+12.5 20 + 6.25 50 + 1.56	0.5+ 25	200 5 2	400	2 4 16	1
no. 27 *Pseudomonas aeruginosa*	1600	>6400	200	400	50 +50	50 +100 200 + 50	32	∞ ∞	4	4 8
no. 28 *Pseudomonas aeruginosa*	6400	>6400	25	50	50 +12.5	50 + 25	128	∞	2	2

TABLE 5.

Minimum bacteriostatic (MCS) and bactericidal (MCC) concentrations of penicillin (P) in U/ml, kanamycin (K) in γ/ml and of the combination of both in some *Proteus* strains. Ratios between these concentrations of the separate antibiotics and those of the antibiotics in the combination.

Strain	P		K		P + K	P + K	MCS P	MCC P	MCS K	MCC K
	MCS	MCC	MCS	MCC	MCS	MCC	MCS P+K	MCC P+K	MCS K+P	MCC K+P
Proteus mirabilis										
no. 29	50	400	50	100	10 + 1.56	5 + 12.5	5	80	32	8
no. 30	20	200	25	25	2 + 12.5 10 + 0.78	5 + 6.25	10 2	40	2 32	4
no. 31	20	50	25	50	2 + 12.5	5 + 6.25 10 + 1.56	10	10 5	2	8 32
no. 32	10	50	50	50	2 + 3.12	2 + 6.25 5 + 3.12	5	25 10	16	8 16
Proteus vulgaris										
no. 36	20	100	12.5	25		5 + 3.12		20		8
no. 37	20	200	25	50	2 + 6.25	1 + 25 5 + 1.56 20 + 0.78	10	200 40 10	4	2 32 64

DISCUSSION.

In vivo, deviations from the in vitro findings are bound to occur. However, since generally there is a good relation between the in vitro and the in vivo activity of antibiotics, and since no other means are available to demonstrate a synergism between two antibiotics, we believe that our findings warrant some conclusions regarding the applicability in vivo. In investigations of this type, however, it should always be borne in mind that conclusions are subject to this restriction.

Both in penicillin-sensitive and in penicillin-resistant *Staphylococcus aureus* we were able (like JADIN (8)) to demonstrate a synergism between penicillin and kanamycin. The average minimum bactericidal concentration of kanamycin (in combination with penicillin) against penicillin-resistant *Staphylococcus aureus*, however, was 1/4 of that of kanamycin alone in our experiments, instead of the 1/8 found by JADIN. In practice it will certainly be possible to make use of this synergism. By simultaneous administration of penicillin in relatively large doses (2-6 million U/day), the kanamycin dosage can be considerably reduced with, consequently, a reduced risk of toxic symptoms.

The effect of the combined antibiotics on *Enterococcus* was variable. If use is to be made of a synergism which, according to results obtained, does not always occur, then the fact remains that concentrations of both antibiotics are required which, at a normal dosage, can only be attained in the urine.

A synergism between penicillin and kanamycin was also observed in a few strains of *E. coli* and *Klebsiella*. Application of the combination in the treatment of infections with these bacteria should apparently be limited to cases showing resistance to other antibiotics and chemotherapeutics, and in which a synergism between the two antibiotics in attainable concentrations has been demonstrated in vitro. Here, too, only infection of the urinary tract could ever constitute an indication.

In view of the observations mentioned under f, it cannot be expected that infections with *Pseudomonas aeruginosa* would respond to treatment with the combined antibiotics.

The results of the tests with six *Proteus* strains are remarkable. An unmistakable synergism was demonstrable in all cases (see also ANDRIEU *et al.* (1)), which would certainly seem to be useful in practice, as in the case of penicillin-resistant *Staphylococcus aureus*, because it permits a lowering of the kanamycin dosage. The range of indications is likely to be chiefly found in urinary infections.

In the case of infections with multi-resistant bacteria which, in vitro, are sensitive to the combination of the two antibiotics, it can be justifiable to make use of this fact even if a normal kanamycin dosage is required to attain the required concentration. The minimum inhibitory concentrations of the two antibiotics separately can be far above the values attainable at normal dosage in these cases.

The results of our investigation also tend to show that the various bacteria are widely different as to ratio of concentrations of the two antibiotics required for a bactericidal effect. Too low a dosage of either antibiotic entails a risk of therapeutic failure and development of resistance; too high a dosage is in many cases incompatible with the purpose of combining the antibiotics, *viz.*, avoidance of toxic reactions to kanamycin administration. For these reasons we feel we must object to the use of quantitatively fixed combinations of the two antibiotics.

Therefore in vitro determination of resistance is desirable before the two antibiotics are used in combination, in order to determine whether and at which dosage combined therapy can be expected to yield results.

Summary.

In vitro, a synergism was demonstrated between kanamycin and penicillin against penicillin-sensitive and penicillin-resistant *Staphylococcus aureus*, and against six *Proteus* strains. Strains of *E. coli*, *Klebsiella*, *Pseudomonas* and *Enterococcus* showed a variable response to the combination of these two antibiotics.

On the basis of the results obtained, a number of conclusions were reached with regard to the range of indications for treatment with these combined antibiotics. Indications include infections caused by penicillin-resistant *Staphylococcus* and urinary infections caused by *Proteus* bacteria. Infections by other bacteria may also constitute an indication, but it is maintained that in vitro determination of resistance is indispensable in all cases.

A number of arguments are presented against the use of a quantitatively fixed combination of the two antibiotics.

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THE RELATION OF PHAGE PATTERN AND LYSOGENICITY IN THE PHAGE TYPING OF STAPHYLOCOCCI OF PHAGE-GROUP II

by

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INTRODUCTION.

Phage typing has become a well established method for the identification of coagulase positive staphylococci, which has rendered extremely valuable services to the epidemiology of staphylococcal infections. With about 25 typing phages and several degrees of intensity of positive reactions the number of possible patterns is extremely large. Due to the relationship between phages the actual number of patterns is more limited and the patterns fall into four main groups. Within each group some patterns are well defined and relatively stable, in other cases the patterns are more variable. Very often different strains show small differences and the different patterns seem to merge into each other.

Theoretically the origin of a phage pattern is not well understood. A strain which does not react with a certain phage might be insensitive (no adsorption of phage) or immune (carrying a related prophage) (ROUNTREE 1956, 1959). As all phages are adsorbed to all staphylococci (HENRY, 1946) the phage patterns are supposed to depend on immunity and not on insensitivity. This has not been proved however. WAHL and FOUACE (1954, 1958) suggest that within their groups the patterns are due to lysogenicity. ROSENBLUM and DOWELL (1960) showed that strains of the WILLIAMS and RIPPON (1952) typing scheme generally carry phages active on strains within the same group. No well defined patterns due to lysogenicity were established however. The cause of immunity against phages from other groups is not understood. Whether defect lysogenic strains of staphylococci exist has hardly been studied.

A second difficulty is the interpretation of the different intensity of positive reactions. What does it mean when a phage which at routine test dilution (1 RTD) shows confluent lysis on the propagating strain gives only ten or a hundred plaques on the strain under test. Though such a low plating efficiency must finally be due to differences between propagating strain and the strain under test it is difficult to explain the mechanism in terms of strain differences. Of course a higher percentage of abortive infections or a higher percentage of lysogenization would produce a lower yield of phage but not a smaller number of plaques. Differences in cell wall receptor or in immunity could explain the phenomenon if only a small fraction of the phage was permitted to enter or to develop. Consequently a low "plating efficiency" probably means an inhomogeneity in the phage population (presence of mutants or of variations induced by the propagating host strain, presence of contaminating phage from the propagating strain which is always lysogenic or recombination between infecting phage and prophage). In these cases one uses a poor method for testing a bacterial character as the number of variations in the testing phage cannot be controlled even under the best conditions of standardization. Of course with the same batch of test phage (containing a certain fraction of variants) weak positive reactions due to variants can prove the identity of two strains of staphylococci and in fact these weak positive reactions proved useful in practice (Ruys and Borst, 1959). Though the empirical value of these weak reactions in phage typing is granted it is unsatisfactory that the explanation is so doubtful.

In the hope to arrive at a better understanding of some of these problems the lysogenicity of a number of strains of staphylococci was studied. In agreement with Rosenblum and Dowell (1960) we found that most strains carried phages, which were only active against strains within their own group. We then concentrated on strains of group II. One of the practical results of this study was the isolation of a number of phages, propagated on a non lysogenic strain, which seem to have more specific host ranges than the standard phages of group II. Consequently weak positive reactions were rare. With the use of these four phages group II can be divided in a relatively small number of distinct patterns which indeed depend mainly on lysogenic immunity. The experiments which lead to the choice of these phages will be described in the present paper.

METHODS.

Phage typing. All strains were kept in stab cultures on meat agar in Byou bottles at room temperature. Before use they were streaked on bloodagar plates and one colony was inoculated in Difco Nutrient Broth. To determine the phage pattern of a strain, agar plates (Difco nutrient agar with 87.6 mg% calcium-chloride) were flooded with broth cultures (16 h 30°C.), drained, spotted with phages and incubated overnight. Reactions were recorded as follows:

++++ = confluent lysis, clear hole in bacterial growth
+++ = confluent lysis, turbid hole (either secondary growth by lysogenization or not completely confluent lysis)
++ = > 100 plages
+ = 10–100 plages
± = < 10 plages
∓ = only very few plages.

Propagating strain. Strain 879 was used as propagating strain for all phages. At first it was thought to be non lysogenic because it was sensitive to all the phages studied and because after irradiation and spotting on 100 strains belonging to group II and on the 25 propagating strains of the standard system and on about hundred strains of other groups it never produced a reaction. On further testing on fifty other strains (group II) two cases showing weak reactions with tiny plaques were observed. Though the strain is thus not really non lysogenic the carried phage could only influence the results in exceptional cases. Thus for practical purposes the strain can be considered as non lysogenic.

Nomenclature. Strains are designated with numbers. The phages derived from these strains are indicated by the same number. As the propagating strains 3^{A}, 3^{B}, 3^{C}, 55 and 71 (N.C.T.C. no's 8319, 8321, 8327, 8358, 9315 resp.) from the standard set were included in this study, the lysogenic phages from these strains will be called L 3^{A}, L 3^{B}, L 3^{C}, L 55 and L 71 to distinguish them from the standard phages.

Carried phages are indicated in parenthesis after the strain number by the number of the phage and the greek character of the phage group. Strain 879 (γ 756) indicating strain 879 which has been artificially lysogenized with phage 756 from the γ group. However strain 269 carrying a natural δ phage which should be designated as 269 (δ 269) is named 269 (δ) for short.

Demonstration of lysogenicity by irradiation with U. V. light. One droplet ($^1/_{30}$ ml) of an overnight broth culture (30°C.) was inoculated into 2 ml broth in a petri dish, incubated for 2 hours at 37°C. and irradiated with ultraviolet light for 3 minutes (Philips Hp 125 W type 103102 lamp, 20 cm distance). After irradiation the culture was incubated for 1 hour at 37°C. and centrifuged for 15 minutes at 3000 rpm. The supernatants were spotted on plates as usual. The amount of phage in the supernatant varied from 10^5—10^8 phage particles per ml. In many cases the yield was 100 times higher than without irradiation. In later experiments the phages were isolated from single plaques, propagated on suitable strains and used at defined concentrations (1 RTD, 5 RTD etc.).

Lysogenization. The strain to be lysogenized was flooded on a plate and spotted with 5 RTD of the lysogenizing phage. Growth in the clear zone of lysis was scraped off and spread on plates. Isolated colonies were picked and tested for immunity against the lysogenizing phage by streaking across a streak of phage (5 RTD) and for lysogenicity by cross-streaking against strain 879 or another suitable strain sensitive for the phage under study. The phage pattern of the lysogenized strain was then established in the usual way. Separate colonies isolated from the same lysogenization experiment generally had the same characters and the same phage pattern. Some exceptions were observed however.

RESULTS.

A. **Lytic spectrum of phages carried by group II strains.**

1. 24 strains from group II were irradiated and spotted on all 25 propagating strains from the standard series. Three strains were non lysogenic (by this test). The other 21 strains produced phages which were active against one or more (generally four) of the five propagating strains of group II. Seven strains gave a positive reaction with propagating strain 73 (group III). Apart from a few occasional plaques on propagating strain 44 no reactions with other propagating strains outside group II were observed. There was no apparent relation between the host-range of the carried phage on

the five propagating strains in group II and the phage pattern of the carrier strain.

We concluded from this experiment that group II is a well defined group, being sensitive to the standard phages of group II and carrying phages active only against strains of group II. This system seemed useful for further study.

2. 48 strains of group II were irradiated and the supernatants spotted on all the 48 strains. The results of the experiment were rearranged as presented in table 1 and suggested some kind of order. Keeping in mind that these results were obtained with supernatants of undefined strength containing perhaps more than one phage one may overlook some discrepancies. Accepting this, the table can be divided in a number of groups as indicated by the lines. The strains of the first group (except strain 268) are non lysogenic (in this experiment). The strains from the group at the bottom carry phage(s) active against a large number of strains. The groups in between contain strains carrying phages with a similar lytic spectrum. Starting from the supposition that these phages would be equal or related the groups were marked α, β, γ, δ, ε and the miscellaneous group ζ. This supposition is supported by the cross resistance within the groups. For instance the δ phages are inactive against all strains carrying δ phages (the noughts in table 1 are in columns which are blank for that group). This is also true for the other groups except for the miscellaneous group ζ.

B. **Isolation of phages.**

1. Phages of the δ group.

The strains from the δ group (269, 764, 334, 681, 763) were irradiated. The supernatant contained between 1—5 × 10^7 phage particles/ml apart from strain 764 which yielded only 3 × 10^5 phage particles. The phages were titrated on strain 879. After isolation from single plaques the phages were propagated on strain 879, filtered and brought to the same titer as the original supernatants. These purified phages had the same lytic spectrum on the 48 strains as the original crude supernatants (compare table 1) barring some minor differences on strains 240, 251, 294 and 78. This shows that the supernatants in the first experiment contained only one phage and consequently that the strains produced only one phage on irradiation. Furthermore the five δ phages were propagated on strain 879, diluted to 1 RTD and 5 RTD and tested on all 48 strains. The lytic spectrum

	3^B	317	363	733	879	176	850	270	245	358	756	184	780	84	246	285	717	284	648	652	273	491	356	509	896	788	3^C	55	3^A	71	269	764	334	681	763	506	268	504	251	294	240	78	355	446	734	706	345	
734																																													○			
345																																															○	
648																			○																													
879					○																																											
78																																										○						
240																																									○							
L 3^A																													○	+																		
268				■			+			/	■										■	■		■	■												○											
363	+		○																									+		+																		α
733	+			○																								+		+																		
652	■																			○							■		+	■																		
356	■	+	■	■	■		■																			/	+	+	+										+									β
850	■	+	■	■	■		○																			+	■	■	■	■																		
270	■	+	■	■	■			○																	+		■	■	■	■																		
245	■	■	■	■	■				○																+	/	■	+	■	+																		
358	■	■	■	■	■					○																+	+	+	■	+									+									
756	■	■	■	■	■	■					○													■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				+						γ
184	■	■	■	+	■	■						○												■	■	■	■	■	■	■	+	■	■	■	■	+	+	+										
780	■	■	■	■	■	■							○							+				■	+	■	■	■	■	■	■	■	+		■	■	+	+			■	■						
84	■	■	■	■	■	■			■					○										■	■	■	■	■				+	+	+	+	+	+	+	■	■	■	+						
246	■	■	■	■	■	■									○									■	■	■	■	+	/		/	+	/		/	+		/	■	■	+	+						
285	■	■	■	■	■	■										○								■	■	■	■	■	+		/		/		/							+						
269	+	■	■	■	■	■	■	■	■	■	■	■	■			■					■	■	■	■	■	■	■	■	■		○								■	■	■	+						δ
764	+	■	■	■	■	■	■	■	■	■	■		■								■	■	■	■	■	■	■	■	■			○									■	■						
334	+	■	■	■	■	■	■	■	■	■	■	■	■								■	■	■	■	■	■	■	■	■				○								■	+						
681	+	■	■	■	■	■	■	■	■	■	■	■	■								■	■	■	■	■	■	■	■	■					○							■	+						
763	+	■	■	■	■	■	■	■	■	■	■	■	■							■	■	■	■	■	■	■	■	■	■						○						■	■						
273	■	■	■	■	■	■	■	■	■	■	■	■	■	+	+	■	■				○		■			+	/		+		■	■	■	■	■	■	■	■	■	■	■	+						ε
491	■	■	■	■	■	■	■	■	■	■	■	■	■	+	+		■					○	■			■		+	■	■	■	■	■	■	■	■	■	■	■	■	■	+						
509	■	■	■	■	■	■	■	■	■	■	+		■	+	+		■							○				+	■	■	+	+	+		+	+	+	+	+		■	+						
896	■	■	■	■	■	■	■	■	■	■	■						■								○					+	■	■	■	■	■	■	■	■										
L 3^C	■	■	■	■	■	■	■	■	■	■	■	■	■	+	+		■										○			■	■	■	■	■	■	■	■	■		■	+							
L 55	■	■	■	■	■	■	■				■	■	■	■	■	■	■					■						○		■	■	■	■	■	■		■	■	■	■	■	+						
284	■	■	■	■	■	■	■	■	■	■	+					+	■	○					■	+	+	+	+	+		■	+	■	■	+	+	■	+	■	■	■	+	+						ζ
294	■	■	■	■	■	■			/	+	■	■	■	■	■	■		+						■	■	■	■	■	/	■	■	■	■	■	■		■	+		○	■	+						
251	■	■	■	■	■	+		+	+	+	■	■	■	■	■	■		■	+	+	+	+	+	■	■		■	■	■	■	■	■	■	■	■	+	■	■	○						+			
506		■	+	+	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	○	■		+	+			+	+				
176	■	■	+	+	■	○	■	+	+	+	■	■	■	■	■	■	■	■	■		+	+	+	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■					+			
355	+	+	+	+	■	+	■	■	■	■	■	■	■	■	■	■	■	■	■	+	■	■	■	■	■	■	+	+		/	■	■	■	■	■	+	■	■	■	+			○					
788	■	■	■	■	■	/	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	○	■	■	■	■	■	■	■	■	■	■	■	■	■	■							+	
446	■	+	/	+	■	+	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	+	■	+	+	+	■	■	■	■	■	+	■	+	+	+				○				
L 71	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					■	■	■	■	■	■	■	■	+	○	■	■	■	■	■	■	■	■	■	■	■	+	■					
L 3^B	○	■	■	■	■	■	+	+	+	+	■	■	■	+	■	■	+	+	+	+	+	+	+	■	■	■	■	■	+	+	■	+	■	■	■		■		■	■	■	+		■		+		
717	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		+		■	■	■	■	■	■	■	■	■	■	+	■	■	■	■	■	■	■	■	■		■	■	■	+				
317	■	○	■	■	■	■	■	■	■	■	■	■	■	■	■	■	+	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					+	■	■	■		
504		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	○	■	■	■	+	■	■		■		

TABLE 1.

The strains are arranged from left to right. The crude phages induced by irradiation of these strains are arranged from top to bottom. Horizontal lines show the lytic spectrum of the phage, vertical columns the pattern of the parent strain.

Strong reactions (+ + + + and + + +) are indicated by ■. Weak reactions (+ + and +) by +. Very weak reactions (±) by /. Negative reactions are left blank.

The noughts indicate the reaction of the phage with the parent strain which is of course negative. The greek characters indicate groups of (crude) phages with comparable lytic spectrum. Within these groups the parent strains show cross resistance (the noughts always fall in blank rows). This does not hold for group ζ. See text.

of the five phages was virtually identical (table 2 second part). In fact some of the discrepancies which were observed in table 1 had disappeared. The five parent strains showed cross immunity against the five δ phages (middle black quadrangle in table 2).

Strain 879 was lysogenized with the five δ phages and the resulting strains proved immune against the five δ phages (table 3 second part). Again, strain 245 sensitive for all δ phages was lysogenized with δ 269 and proved immune to all δ phages (table 6).

It can thus be argued that the staphylococcal strains in group δ carry one phage each, that these phages have virtually the same lytic spectrum, that these phages are inactive against the other four strains in the δ group and inactive against strains which have been lysogenized with a δ phage. For practical purposes these phages will at the moment be considered identical or very much related. The phage patterns of the δ lysogenic strains will be discussed later.

Phage δ 269, isolated and propagated on the non lysogenic strain 879 was chosen as the δ phage for typing purposes.

2. Phages of the γ group.

Already in table 1 it can be seen that the γ group is not quite homogeneous. The lytic spectrum of the first three phages (756, 184 and 780) differs slightly from that of the second three.

The six strains of the γ group were irradiated, the supernatants titrated on strain 879 and the six phages were isolated from single plaques and propagated on strain 879. The lytic spectrum of these six phages at 1 and 5 RTD (table 2) were very similar to those of the crude phages in table 1 suggesting that each strain carried only one phage. In these lower dilutions however the reactions with strains of the δ group and part of the ε group were weak or negative with the exception of phage 756 which reacted well with all δ strains. Arbitrarily the γ from 756 will be called γ_1, the others γ_2.

Some chance observations confirm that the strains carry only one phage. Strain 84 which at the first irradiation yielded a γ phage produced no phage in a later experiment. After plating out from the stock culture, two colony-types were observed one with smaller, one with larger haemolytic zones. The colonies with large haemolytic zones were non lysogenic and sensitive to all fifteen γ, δ and ε phages. The colonies with smaller haemolytic zones were lysogenic yielding a γ_2 phage and were insensitive to the five γ and the five δ phages (at 1 RTD). The same phenomenon was observed with

TABLE 2.

PHAGES ↓	STRAIN NUMBER OF STAPHYLOCOCCI AND TYPE OF PHAGE CARRIED																																													
	879	317	251	756	184	780	84	246	285	717	294	269	764	334	681	763	268	506	504	71	356	273	491	896	509	3^C	55	245	270	358	850	363	733	176	3^B	3^A	788	648	284	734	345	706	240	78	355	446	652
				γ	γ	γ	γ	γ	γ	ζ	ζ	δ	δ	δ	δ	δ	δ	ζ	ζ	ζ	ε_2	ε_2	ε_2	ε	ε_1	ε_1	ε_1	β	β	β	β	α	α	ζ	ζ												
γ_1 756	++++	++++	++++	–	–	–	–	–	–	–	–	++++	++++	++++	++++	++++	++++	++++	++++	++++	–	–	–	++++	+++	++++	++++	–	–	–	–	+++	++++	++++	++++	+	++++	–	–	–	–	–	–	+	–	–	–
γ_2 184	++++	++++	++++	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	++++	–	–	–	∓	++++	++++	++++	–	–	–	–	–	–	++++	++++	–	++	–	–	–	–	–	–	–	–	–	–
780	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	∓	++++	++++	++++	–	–	–	–	–	–	++++	++++	–	++	–	–	–	–	–	–	–	–	–	–
84	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++++	+++	++++	–	–	–	–	++++	++++	++++	++++	–	++++	–	–	–	–	–	–	–	–	–	–
246	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	∓	–	±	–	∓	–	–	–	–	–	++	++++	++++	+++	–	–	–	–	+++	+++	+++	++++	–	++++	–	–	–	–	–	–	–	–	–	–
δ 269	++++	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	+++	++++	++++	++++	+++	++++	+++	++++	++++	++++	++++	++	++	++++	∓	+	++++	–	–	–	–	–	–	–	–	–	–
764	++++	+++	+++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++	++	++++	–	+	++++	–	–	–	–	–	–	∓	–	–	–
334	++++	++++	++++	++++	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++++	+++	++	++++	–	+	++++	–	–	–	–	–	–	±	–	–	–
681	++++	++++	++++	++++	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++++	–	+	++++	–	–	–	–	–	–	+	–	–	–
763	++++	+++	+++	+++	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	+++	+++	+++	++++	+++	++++	++++	++++	++++	++	++	++++	–	+	+++	–	–	–	–	–	–	∓	–	–	–
ε 509	++++	++++	++++	+	–	–	–	–	–	++	++	±	+	±	–	–	±	+	?	+	–	–	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–
273	++++	++++	++++	+++	+	+	–	–	–	+++	++	++	+	+	++	++	+	++	+++	++	–	–	–	–	–	–	–	++++	++++	++++	++++	+++	+++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–
491	++++	++++	++++	+++	+	++	–	–	?	++++	++	++	++	++	++	++	++	++	+++	++?	–	–	–	–	–	–	–	++++	++++	++++	++++	+++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–
L3^C	++++	++++	++++	+++	–	++	–	++	[+++]	++++	–	+++	+++	+++	++++	+++	+++	++++	++++	++++	–	–	–	–	–	–	–	++++	++++	++++	++++	++++	++++	++++	++++	–	–	[+++]	–	–	–	–	+	++	–	–	–
L55	++++	++++	++++	++++	++++	++++	+++	++++	++++	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–	+	+	–	–	–
71	++++	+++	+++	+++	++++	+++	+++	++++	++++	+++	+++	+++	++++	++++	+++	++++	++++	++	++	++++	+++	++++	++++	++++	+++	++++	++++	++++	++	+++	++++	–	–	–	–	+	–	+++	+++	–	–	–	–	–	–	–	–

strain 184. In both cases a variation from lysogenic to non lysogenic was observed which could hardly be due to the simultaneous loss of two phages.

The phages of the γ group are obviously related. Notwithstanding the differences between γ_1 and γ_2 the lytic spectra of the six phages are very similar. None of these phages react with the six strains carrying γ phages.

Strain 879 was lysogenized with several phages from this group. The resulting strains proved resistant to all γ phages (table 3 first part). Again, strain 509 sensitive to all γ phages after lysogenization with phage 756 γ_1, proved resistant to the other five γ phages (table 7). Consequently we consider the phages of the strains in the γ group as related phages.

Phage 756 (γ_1) was again purified from a single plaque, propagated on strain 879 and used as the γ phage for typing purposes.

3. Phages of the ε group.

The ε group was treated in the same way. The six strains of the ε group were irradiated, the phages isolated and propagated on strain 879. The lytic spectra of these phages (at 5 RTD) are shown in table 2. They are comparable to those of the crude phages in table 1 indicating that each strain carried only one phage. The lytic spectra of four of the five phages are more or less equal though there are some differences with regard to γ strains. The spectrum of the fifth (L 55) differs with regard to β lysogenic strains.

Strain 879 was lysogenized with several ε phages. The resulting strains proved immune against all ε phages (table 3). The same was true for strain 176 lysogenized with ε 273 (table 6).

The equality of the lytic spectra and the cross immunity suggest that the five ε phages are related. Nothwithstanding its aberrant

TABLE 2. Lytic spectrum of isolated phages at 5 RTD.

The host range (lytic spectrum of the phages) can be read from left to right. The phage pattern (sensitivity and immunity) of the parent strain is seen from top to bottom. The lytic spectrum of the five δ phages is nearly equal. The parent strains of the δ phages are immune to all δ phages (middle black quadrangle), but also against part of the γ phages. The lytic spectrum of γ and ϵ phages is less homogeneous. The immunity of γ strains extends to δ phages and to some ϵ phages. The immunity of some ϵ strains to γ phages is due to double lysogenicity. At 1 RTD virtually equal results were obtained.

TABLE 3.

PHAGES →	γ PHAGES						δ PHAGES					ε PHAGES					PHAGE
STRAINS ↓	756.	184.	780.	84.	246.	285.	269.	764.	334.	681.	763.	509.	273.	491.	L3C.	L55.	71
879 (γ756)	−	−	−	−	−		+++	+++	+++	+++	+	+	+	++	++	++++	++++
756 (γ)	−	−	−	−	−		+++	++++	++++	++++	+++	+	+++	+++	+++	++++	++++
879 (γ184)	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	++++	++++
184 (γ)	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	++++	++++
879 (γ780)	−	−	−	−	−	−	−	+	++	++	−	++	+	++	−	++++	++++
780 (γ)	−	−	−	−	−	−	−	?	+	?	−	++	+	++	+	++++	++++
879 (γ 84)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++++
84 (γ)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++
879 (γ246)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	+++
246 (γ)	−	−	−	−	−		−	−	−	−	−	−	−	−	++	++++	++++
879 (γ285)	−	−	−	−	−	−	−	−	−	−	−	++	+	+	+	++++	++++
285 (γ)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	++++
879 (δ269)	++++	+	−	−	−		−	−	−	−	−	∓	++	++	+++	++++	++++
269 (δ)	++++	+	−	−	∓		−	−	−	−	−	±	++	++	+++	++++	++++
879 (δ764)	++++	+	−	−	−	−	−	−	−	−	−	±	+	++	+	++++	++++
764 (δ)	+++	+	−	−	−	−	−	−	−	−	−	+	+	+	+	++++	++++
879 (δ334)	+++	+	−	−	∓	−	−	−	−	−	−	+	+	++	++	++++	++++
334 (δ)	++++	+	−	−	∓	−	−	−	−	−	−	+	+	++	++	++++	++++
879 (δ681)	++++	+	−	∓	±	∓	−	−	−	−	−	+	+	++	+	++++	++++
681 (δ)	++++	+	−	−	∓	∓	−	−	−	−	−	+	+	++	+	++++	++++
879 (δ763)	+++	+++	−	+	+	+	−	−	−	−	−	+++	++	+++	+++	++++	++++
763 (δ)	+++	+	−	±	−	−	−	−	−	−	−	−	++	++	+++	+++	++++
879 (ε509)	+++	+++	+++	+++	+++	+++	++	+++	+++	++++	++	−	−	−	−	−	++++
809 (ε)	+++	++++	++++	++++	++++	+++	+++	+++	+++	+++	++	−	−	−	−	−	++++
879 (ε273)	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	−	−	−	−	−	++++
273 (ε)	−	−	−	−	−	−	+++	+++	+++	+++	++	−	−	−	−	−	++++
879 (ε491)	++++	++++	++++	+++	++++		++++	++++	++++	++++	+++	−	−	−	−	−	++++
879 (β491)	−	−	−	−	−	−	++++	+++	+++	++++	++++	++++	+++	+++	++	−	++++
491 (εβ)	−	−	−	−	−	−	++++	++++	++++	++++	+++	−	−	−	−	−	++++
879 (ε356)	++++	++++	+++	+++	+++	+++	+++	++	+++	++++	+++	−	−	−	−	−	++++
356 (ε)	−	−	−	−	−		+++	++++	++++	++++	+++	−	−	−	−	−	++++
879 (εL3C)	+++	++	+++	++	+++	+++	+++	+++	+++	+++	++	−	−	−	−	−	++++
3C (ε)	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	++	−	−	−	−	−	++++
879 (ε 55)	++++	++++	++++	++++	++++		++++	++++	++++	++++	++++	−	−	−	−	−	++++
55 (ε)	++++	++++	++++	++++	+++		+++	+++	++++	++++	+++	−	−	−	−	−	++++
879 (71)	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++	+++	++	−	−

lytic spectrum ε phage L 55 was chosen as the ε phage for typing purposes (Phage L 55 is of course quite different from the standard phage 55).

4. Phages of the β group.

The β group was treated in the same way. The lytic spectrum of the isolated phages was (with one exception) quite identical and equal to that in table 1. The β phages are probably related to the γ phages as shown by the cross resistance in table 1. As these phages have a very small host range they are not very useful for typing purposes and will not be discussed further. The α group was not studied. The ζ group has not yet been studied intensively. For some of these strains it has been observed that they carry a broad spectrum phage related to phage 71 of the WILLIAMS and RIPPON system. Other strains may be doubly lysogenic, carrying more than one phage. One such case has been observed.

5. Serologic relationship between γ, δ and ε phages.

A serum was prepared against phage γ 756 by the method of ADAMS (1950). After absorption with killed staphylococci of strain 879, the neutralization constant for the homologous phage was 22. All phages of the γ, δ and ε group with the exception of phage L 55 were neutralized to equal extent by this serum and seem to belong to the same serological group.

The antigenic difference between phage L 55 and the other ε phages means that L 55 should be removed from the ε group, which is in accordance with the aberrant lytic spectrum.

TABLE 3. Comparison of the phage patterns of artificially lysogenized strains with the phage pattern of the parent strain of the lysogenizing phage.

The strains are given in pairs. First the pattern of strain 879 after lysogenization with an isolated phage, then the pattern of the parent strain of the phage. In general these patterns are equal showing that lysogenicity determines immunity. The ε phages from strain 273, 491 and 356 produce a different pattern in strain 879, the immunity to γ phages is not transferred.
This is due to double lysogenicity of the parent strains (see text). The second phage from strain 491 was also transferred to 879 producing only the γ immunity.
Lysogenization of the non lysogenic strain 184 with the same phages produced virtually identical results.

The serum did not neutralize phage 71 or phages 3^A, 3^B, 3^C and 55 of the Williams and Rippon system. The γ, δ and ε phages consequently do not belong to the serological groups A and B.

C. **Adsorption experiments.**

It has been shown before that staphylococcal phages are generally adsorbed to most coagulase-positive staphylococci. To exclude the possibility that resistance to certain phages might be due to insensitivity (that is to non adsorption of phages) a number of adsorption experiments was undertaken. The results showed that the phages were always adsorbed to the bacteria, whatever combination (within group II) was chosen. Phages from other groups were not always adsorbed. Of course this type of experiment does not show that the phage always infected the bacteria but supports the view that the resistance is mainly due to "immunity".

D. **Phage typing with the new phages.**

If the reasoning that γ, δ and ε phages formed definite groups was sound it should be possible to obtain more definite phage patterns within group II by typing with these phages. Phages γ 756, δ 269 and ε L 55 were chosen for this purpose. As many strains would not be sensitive to one of the phages of these groups it seemed necessary to add a broad spectrum phage from the ζ group (table 1). Some of the phages of group ζ are related to phage 71 from the standard typing system. Standard phage 71 was therefore used.

To eliminate the possible interference of lysogenic phages from propagating strain 71, phage 71 was propagated on strain 879. It has been shown with 48 strains of group II that the reactions of phage 71 produced on strain 71 or on 879 are equal. The four phages γ 756, δ 269, ε L 55 and 71 were all propagated on the same strain 879, brought to 1 RTD, 5 RTD and 200 RTD and 143 strains of staphylococci from phage group II were tested with these phages. The reactions with 1 RTD and 5 RTD were virtually equal. Even with 200 RTD the patterns were the same. Only phage ε L 55 acted on more strains at 200 RTD than at lower dilution. An instance of the results is given in table 4, together with the results of phage typing with the standard phages of group II.

A summary is given in table 5. Eighty percent of all strains can be allocated to about ten distinct patterns which seem related to lysogenicity (see below); three strains are missed with 1 RTD,

TABLE 4.

Phage patterns with the new phages and with standard phages.

The new phages are γ 756, δ 269, ϵ L 55 and 71 (propagated on 879). The reactions left of the vertical line give the pattern according to the new system. The pattern with the standard phages is to the right of the line (in inversed order). The reaction with phage 71 (left of the line) belongs to both systems.

Strains which have been analysed for lysogenicity are indicated as such. All reactions are given with 1 RTD except for group N which contains strains which are non typable with both systems. In this case strong phages (200 or 1000 RTD) were used.

Strain	phage								remarks
	γ	δ	ϵ	71	55	3^C	3^B	3^A	
879	++++	++++	++++	++++	++++	++++	++++	—	pattern A
317	++++	++++	++++	++++	++	+	+	+	
251	++++	++++	++++	++++	+++	+	++	—	
921	++++	++++	++++	++++	+	++++	+++	—	
939	++++	++++	++++	++++	++++	++++	++++	+	
947	++++	++++	++++	++++	++++	++++	++++	+	
R 7	++++	++++	++++	+++	++++	++++	+++	+	
R 10	++++	++++	++++	++++	++++	++++	++	+	
756 (γ)	—	++++	++++	++++	++	++	++	—	pattern B γ lysogenic(γ_1)
958	—	++++	++++	++++	++	+++	+++	±	
1170	—	++++	++++	++++	++++	++	++++	±	
R 9	—	+++	++++	++	++++	++++	++++	+	
R 41	—	++++	++++	++++	++++	++++	+	+	
R 47	—	++++	++++	++++	++++	++++	+	+	
1019	—	++++	++++	++++	—	—	—	—	
1344	—	++++	++++	++++	±	—	—	—	
1348	—	++++	++++	++++	—	—	—	—	
84 (γ)	—	—	++++	+	+++	++	+++	+	pattern C γ lysogenic(γ_2) with extension of the immunity to δ phages
246 (γ)	—	—	++++	+++	++	+	++	—	
717 (γ)	—	—	++++	++	—	—	—	—	
285 (γ)	—	—	++++	+++	++	+	++	±	
184 (γ)	—	—	++++	+++	++	++	++	++	
780 (γ)	—	—	++++	+++	++++	++	+++	±	
294	—	—	++++	+++	++	+	++	—	
648	—	—	+++	++	+++	+	—	—	
1165	—	—	++++	++++	++	++	+++	±	
1212	—	—	++++	++	++	+	+	++	
71	++++	—	++++	+	+	+	—	—	pattern D δ lysogenic
269 (δ)	++++	—	+++	+++	+++	++	++	—	
334 (δ)	++++	—	+++	+++	++	++	++	—	
506	++++	—	++++	+	+++	+++	+++	—	
763 (δ)	++++	—	+++	+++	++	+++	++	±	
764 (δ)	++++	—	+++	++	++	+++	++	±	
268	++++	—	+++	+++	+++	++	++	—	
681 (δ)	++++	—	+++	++	++	++	++	—	
932	++++	—	++++	++++	++	+++	+++	—	
1205	++++	—	++++	++++	++	++	++	±	
509 (ϵ)	++++	++++	—	++++	+++	+++	+++	—	pattern E ϵ lysogenic
896 (ϵ)	++++	++++	—	+++	++	+++	+++	—	
3C (ϵ)	++++	++++	—	++	++	++	+	—	
55 (ϵ)	++++	++++	—	+++	+++	+++	+++	±	
933	++++	+++	—	++++	++	+++	+++	±	
1402	+++	+++	—	++++	+++	++	+++	+++	
R 16	++++	++++	—	++++	++++	++++	++++	+	
R 23	++	+	—	+++	++++	++++	+++	++	

TABLE 4 (*continued*).

Strain	phage								remarks
	γ	δ	ε	71	55	3^C	3^B	3^A	
273 (ε)+β	—	++++	—	+++	++	—	—	—	pattern F β or (ε + β) lysogenic (pure 71 of the standard system)
491 (ε)+β	—	++++	—	+++	—	—	—	—	
356 (ε)+β	—	++++	—	++	—	—	—	—	
981	—	++++	—	++++	—	—	—	—	
989	—	+++	—	++++	—	—	—	—	
995	—	+++	—	++++	—	—	—	—	
1392	—	++++	—	++++	—	—	—	—	
1434	—	++	—	++++	—	—	—	—	
1468	—	+++	—	++++	—	—	—	—	
1483	—	+++	—	++++	—	—	—	—	
245 (β)	—	++++	—	++++	—	—	±	—	
270 (β)	—	++++	—	++++	—	—	—	—	
358 (β)	—	++++	—	+++	—	—	—	—	
850 (β)	—	++++	—	++++	—	—	±	—	
284	—	—	—	++	±	—	—	—	pattern G
1343	—	—	—	++++	—	—	±	—	
1350	—	—	—	++++	+	+	—	—	
1425	—	—	—	++++	+	+	—	—	
1491	—	—	—	++++	+	++	—	—	
R 1	—	—	—	++++	—	—	—	—	
R 35	—	—	—	++++	+	++++	—	—	
R 46	—	—	—	++++	+	+++	—	—	
176	++++	++++	++++	—	+	++	+	—	pattern H
363	++++	++++	++++	—	++	—	—	—	
733	++++	+++	++++	—	++	+	+	—	
3^B	+++	+	++++	—	+	++++	++++	—	
R 31	+++	++	++++	—	+	++++	+	—	
924	—	—	++	—	—	+	+	++++	pattern K
927	—	—	++++	—	+	++++	++++	±	
1395	—	—	++++	—	++++	++	+++	+	
R 4	—	—	+	—	+	++	—	+++	
R 6	—	—	++++	—	++++	++++	+++	++	
R 13	—	—	++++	—	++++	++++	+++	++++	
50	—	—	+	—	—	—	—	—	group N Non typable strains tested with strong phages.
734	—	—	++	—	++	—	—	—	
345	—	—	++	—	—	—	—	—	
706	—	—	+++	—	++++	++++	+++	—	
240	—	—	+++	—	+++	++	—	++	
78	—	—	+++	—	+	±	±	+++	
355	—	—	+++	—	+++	+++	+++	—	
446	—	—	+++	—	+++	+++	+++	—	
652	—	—	+++	—	++++	++++	++++	—	

two of these react however with 200 RTD. Apart from the 134 strains in table 6 nine strains which could only be typed with concentrated phages of the standard system were again only typable with 200 RTD of the new set of phages.

It is remarkable that weak reactions are rare and that the patterns are much more distinct than those with the standard system (table 4). Pattern F is obviously "pure 71" according to the standard system. As was to be expected the correlation between the patterns of the standard system with the new system is incomplete (f.i. the occurrence of "pure 71" in the new patterns B and C).

TABLE 5.

Observed patterns by typing with the new phages.

Number of strains	reaction with phages				pattern	lysogenicity	
	γ	δ	ϵ	71		probable	proved for
13	++++	++++	++++	++++	A	non lysogenic?	
9	—	++++	++++	++++	B	γ_1 lysogenic	1 strain
18	++++	—	++++	++++	D	δ lysogenic	5 strains
11	++++	++++	—	++++	E	ϵ lysogenic	4 ,,
7	++++	++++	++++	—	H	71 lysogenic?	
27	—	—	++++	++++	C	γ_2 lysogenic	6 ,,
24 1)	—	++++	—	++++	F	β or $(\epsilon + \beta)$ lysogenic	7 ,,
8	—	—	—	++++	G	double lysogenic ?	
7	—	—	++++	—	K	?	
3	++++	—	++++	—	L	?	
2	+++	++++	—	—			
1	+++	—	—	++++			
3	—	—	—	—	M	?	

1) Nineteen strains proved β lysogenic, five strains lysogenic for $(\epsilon + \beta)$.

All things taken together we think that the new set of phages permits a distinct differentiation of pattern A to K within group II. A further advantage of the system is that the four phages are propagated on the same strain which is for practical purposes non lysogenic. Finally (or perhaps consequently) weak reactions with these phages seem to be rare.

The distinct patterns suggested a definite relation between lysogenicity and phage pattern. Patterns B, D, E and H (table 5) would then be due to lysogenicity for phages γ, δ, ε and 71 respectively (45 strains), the patterns below the horizontal line in table 5 due to double lysogenicity. The results seemed to indicate that a further study of phage pattern and lysogenicity might be fruitful.

E. The phage pattern (resistance pattern) of the lysogenic parent strains.

1. δ lysogenic strains.

According to the hypothesis, that lysogenicity determines the phage pattern, the parent strains of the δ phages should be immune to all δ phages and sensitive to the phages of other groups (γ and ε). Table 2 (read from top to bottom) shows that the lysogenic strains are indeed immune against δ phages but also against most phages of the γ group.

One might save the hypothesis by supposing that the δ strains carry also a (defective) γ prophage. This is not the case because most of the δ lysogenic strains prepared from strain 879 by lysogenization with a pure δ phage are also immune to most γ phages (table 3, second part, left to right). The presence of a δ prophage therefore confers an immunity to (or interferes with) some γ phages (but not to γ 756 used in the new typing set).

We think that the immunity conferred by the δ phage on the staphylococcal strains extends to part of the γ phages, probably because the cytoplasmatic suppressor (JACOB and CAMPBELL, 1959) induced by the δ prophage also inhibits the development of γ phages. Whether one wants to call this co-immunity (cross-immunity) or interference depends on the question whether one considers γ and δ phages as different or related and is purely a question of semantics. The important point is that the pattern of the δ strain is due to their prophage.

2. γ lysogenic strains.

The phage pattern of the six strains of the γ group is not homogeneous. Table 1 shows that strains 756, 184 and 780 are insensitive to γ phages and sensitive to δ phages whilst the other three strains (84, 246 and 285) are immune against both γ and δ phages. The presence of some γ prophages seems to confer an immunity to δ phages whereas others do not. The presence of a γ prophage even seems to confer an immunity against some ε phages (table 2). From table 3 it can be derived that the phage pattern of strains of 879 artificially lysogenized with pure γ phages is generally equal to that of the corresponding natural strains and that the immunity extends to some δ and ε phages (but not to phage ε L 55 of the new typing set).

It was at first believed that the immunity of γ strains to δ phages might not be absolute and dependent on phage concentration. F.i. strain 184 (γ) was sensitive to the crude (and concentrated) δ phages (table 1) but immune to the purified δ phages at 1 and 5 RTD in table 2 and 3. To test whether intra-group immunity could be distinguished from extra-group immunity (interference) with higher phage concentrations several γ and δ lysogenic strains were tested with δ and γ phages at 200, 20, 2 and 0.4 RTD. All strains that were immune against a phage at 2 RTD were also immune at 200 RTD. It is consequently thought that the degree of immunity and the degree of interference are both relatively independent of the phage concentration. The discrepancy between the results with strain 184 (γ) in table 1 and in later experiments remains unexplained though it should be remembered that the phages in table 1 were obtained by U.V. irradiation of lysogenic strains whereas in later experiments phages produced on PS 879 were used.

3. ε group.

The staphylococcal strains carrying ε phages fall into two groups, those that are immune against ε phages and those that are also immune against γ phages (table 2). The strains of 879 lysogenized with ε phage (table 3) generally show the same pattern as the parent strain from which the phage was derived. However the patterns of strains 491, 273 and 356 differ from the corresponding strains of 879. In these cases the immunity to γ phages is not transferred to 879. This suggests that these strains carry more than one phage.

To prove the double lysogenicity of these strains, strain 491 (ε) was irradiated, incubated for two hours and centrifuged. The supernatant was titrated on strains 879 (sensitive for all phages) and 879 (ε 491) which is insensitive to the phage ε 491. As expected a high titer was observed on strain 879. On strain 879 (ε 491) about 100 plaques (presumably of the second phage) were observed only in the undiluted fluid. One of these plaques was picked in 0.1 ml broth and after centrifugation this fluid was titrated on strain 879. In the dilution 1 in 100 several plaques were observed. Considering that in this limiting dilution contaminating ε phage from strain 879 (ε 491) would be eliminated one of these plaques was picked and used for phage propagation. This phage showed the lytic spectrum of a β phage. Strain 879 lysogenized with this phage was immune towards γ phages and phage L 55 (see table 3, ninth line from bottom). This is the phage pattern of β strains (compare table 2). It is thus concluded that strain 491 is carrying an ε and a β phage. The phage pattern of strain 491 (β, ε) is explained by the presence of two prophages. That the strain was originally classified as an ε strain is due to the fact that after irradiation ε phage is produced in excess.

The same holds for strains 273 and 356. In fact strain 356 was originally classified as a β strain (table 1). On isolation from a single plaque the strain yielded an ε phage however. In this case the β phage was produced in excess with the lytic spectrum shown in table 1. On purification a plaque from the minority phage ε was obviously picked by accident.

We conclude therefore that the ε group can be subdivided in two parts: carrying ε phage and immune to ε phages but sensitive to γ phages or carrying ε and β phage and consequently immune to ε and γ phages.

F. **Doubly lysogenic strains and phage substitution.**

A number of doubly lysogenic strains were prepared by lysogenization of 879 first with one phage and subsequently with another phage or by lysogenization of a strain of known lysogenicity with a second phage. The results are in table 6. The phage patterns of these doubly lysogenic strains seem to corroborate the hypothesis that the patterns are due to lysogenicity. Furthermore they indicate that phages of the groups β and δ, ε and 71, ε and γ and ε and δ are distinct as double lysogenization with identical phages is unusual (see however Bertani, 1954).

With strain 509 (ε) (γ 756) it was shown by irradiation and titration on suitable strains [509 (ε) and 850 (β) see table 2] that about 1000 γ particles were produced against one ε particle. In this case the ε phage was certainly not defective as 509 (ε) produced high titers of ε phage on irradiation.

During lysogenization with a second phage the original (pro)phage may be lost. A number of such instances of phage-substitution were observed (table 7). Strains derived from colonies resistant to the superinfecting phage proved to have lost the immunity due to the original phage. Even in the same experiment some resistant colonies consisted of doubly lysogenic cells whereas other colonies showed substitution.

If the bacterial population of the lysogenic strain contains a fraction of "cured cells" not carrying prophage, such cells may be lysogenized by the superinfecting phage thus imitating the phenomenon of true substitution. Our method of isolating resistant colonies does not distinguish between true substitution and fake-substitution.

It is however remarkable that substitution was rarely observed in most double lysogenization experiments but always occurred when γ strains were lysogenized with δ phages and vice versa. In fact no doubly lysogenic γ, δ strains were obtained. This seems to indicate that phages of the γ and δ group are more closely related than those of the other groups.

TABLE 6.

Doubly lysogenized strains.

The strains are given in pairs. The first strain of each pair is doubly lysogenic. It was obtained by lysogenizing the second strain of each pair with a second phage. The resistance patterns (phage patterns) are given on the horizontal lines. They correspond well with the thesis that the resistance pattern is due to lysogenicity.

	γ phages						δ phages					ϵ phages					phage	
strains	756	184	780	84	246	285	269	764	334	681	763	507	273	491	L3^C	L55	71	
176 (ζ) (ϵ 273)	+++	+++	+++	+++	++++	+++	+++	+++	++++	++++	+	—	—	—	—	—	—	ζ — ϵ
176 (ζ)	++++	++++	++++	++++	++++		++++	++	++	++	++	++++	++++	++++	++++	++++	—	
245 (β) (δ 269)	—	—	—	—	—	—	—	—	—	—	—	+	+	++	+	—	++++	β — δ
245 (β)	—	—	—	—	—	—	+++	++++	++++	++++	+++	++++	+++	+++	++++	—	++++	
509 (ϵ) (γ 756)	—	—	—	—	—	—	+++	+++	+++	+++	++	—	—	—	—	—	++++	ϵ — γ
509 (ϵ)	++++	++++	++++	++++	++++		+++	++++	+++	++++	+++	—	—	—	—	—	+++	
879 (δ 763) (ϵ L55)	+++	+++	—	++	++	+	—	—	—	—	—	—	—	—	—	—	++++	δ — ϵ
879 (δ 763)	++++	+++	—	++	++	++	—	—	—	—	—	+++	+++	++++	+	++++	++++	
879 (ϵ L55) (δ 269)	+++	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	++++	ϵ — δ
879 (ϵ L55)	++++	++++	++++	++++	+++		+++	+++	++++	++++	+++	—	—	—	—	—	++++	

DISCUSSION.

The isolated phages can be ordered, according to their lytic spectrum, into three groups (table 2). Phages of each group are inactive towards strains, which carry a phage from the same group (table 3). This is sufficient evidence to consider the γ, δ and ε phages as more or less distinct groups. Doubly lysogenized strains (table 6) seem to support the differences between most groups. On the other hand the phages are in some way related as is suggested by the few serological data and perhaps by the extension of lysogenic immunity outside the group. The γ and δ phages seem more closely related than the other groups.

Phage typing with four selected phages produced distinct patterns which in 77 out of 134 strains could definitely be ascribed to lysogenicity for one phage (table 5, column 4). Pattern F contains β lysogenic strains as well as strains which are doubly lysogenic for ε and β. Together 101 out of 134 patterns can definitely be related to lysogenicity. A subdivision of pattern F in β lysogenic and doubly lysogenic strains would be possible by the addition of an ε phage to the typing system (see note table 5). Again pattern A can be subdivided with phage 317.

The results of the study of the phage patterns of the lysogenic strains suggest that the phage patterns are indeed mainly due to lysogenicity. There are however some complications:

a. the immunity conferred upon a strain by a prophage of one group does extend in some cases to some phages of other groups even in artificially lysogenized strains. This co-immunity or interference might be due to a suppressor in the cytoplasm.

b. in some cases strains may carry two prophages but on irradiation produce one phage in excess so that the other phage is overlooked. The apparent discrepancy between lysogenicity and phage pattern in strains 491 (ε β), 273 (ε β) and 356 (ε β) disappeared as soon as the double lysogenicity was discovered.

c. we have some weak indications that the immunity of a strain is not always determined by the prophage alone. The same (pro)-phage can produce different immunity patterns in different strains. Strains 285 (γ) and 879 (γ 285) (table 3) are perhaps an instance. Other instances have been observed. This might mean that the host cell contributes by some means to the immunity

TABLE 7.

Substitution of phages.

The strains are given in pairs. The first strain of each pair was obtained by lysogenizing the second strain of the pair with a second phage. The first strain always gained the resistance due to the superinfecting phage but always lost the resistance due to the prophage of the parent strain. The first strain in each pair is consequently not doubly lysogenic but only carries the second phage indicated in parenthesis after the strain number.

	γ phages						δ phages					ε phages					phage	
strains	756	184	780	84	246	285	269	764	334	681	763	509	273	491	L3C	L55	71	
879 (δ **763**) (ε 491)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	—	—	—	—	++++	δ — ε
879 (δ 763)	++++	++++	—	++	++	++	—	—	—	—	—	+++	+++	++++	+	++++	++++	
879 (ε **491**) (δ 269)	++++	++	—	—	—	—	—	—	—	—	—	+	++	++	++	++++	++++	ε — δ
879 (ε 491)	++++	++++	++++	+++	++++		++++	++++	++++	++++	+++	—	—	—	—	—	++++	
879 (γ **756**) (δ 269)	++++	±	—	—	±	—	—	—	—	—	—	±	+	+	+	++++	++++	γ — δ
879 (γ 756)	—	—	—	—	—	—	+++	+++	+++	+++	+	+	+	++	+	++++	++++	
879 (δ **269**) (γ 756)	—	—	—	—	—	—	++++	++	+++	++++	+++	+	++	++	++	++++	++++	δ — γ
879 (δ 269)	++++	+	—	—	—		—	—	—	—	—	±	++	++	+++	++++	++++	
879 (δ **763**) (γ 756)	—	—	—	—	—	—	++++	++	+++	++++	+++	+	+	++	+	++++	++++	δ — γ
879 (δ 763)	++++	++++	—	++	++	++	—	—	—	—	—	+++	+++	++++	+	++++	++++	
879 (δ **764**) (γ 756)	—	—	—	—	—	—	+++	++	+++	+++	+++	+	+	+	+	++++	++++	δ — γ
879 (δ 764)	++++	+	—	—	—	—	—	—	—	—	—	±	+	++	+	++++	++++	

f.i. by influencing the quantity or quality of the cytoplasmatic suppressor which is induced by the prophage.

This suggests that the phage pattern of staphylococci is due to at least two factors:

a. the prophage(s) inducing immunity to phages of the same group or interference with phages of related groups.
b. a host factor determining the intensity or the extension of the immunity or interference.

Summary.

A system of four phages is described which permits the differentiation of staphylococci in phage group II into about ten distinct patterns which are dependent on lysogenicity (see table 5). All phages are propagated on one propagating strain. Weak reactions are rare. The new patterns II A, II B . . . . . . to II K correlated reasonably with the patterns of the Williams and Rippon system.

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(Central Laboratory, Ministry of Economic Affairs, Brussels, Belgium, and Laboratory for Microbiology, Faculty of Sciences, State University, Gent, Belgium).

AN IMPROVED METHOD FOR THE PREPARATION OF 3-KETOGLYCOSIDES

by

M. J. BERNAERTS and **J. DE LEY** [1])

(Received February 7, 1961).

INTRODUCTION.

Previously we described the oxidation of lactose, maltose, lacto- and maltobionic acids to the corresponding 3-ketoglycosides by bacteria provisionally called *Alcaligenes faecalis* (BERNAERTS and DE LEY, 1958, 1960). This process requires a strong aeration and a heavy inoculum with young organisms. It was found that the yield of oxidation products often varied unpredictably from one experiment to the other, particularly with substrates from which low yields were obtained such as *e.g.* maltose.

In the present work several factors have been studied in order to improve the yield of the end products: (1) the rate of aeration, which can be changed within large limits using a fermentor operating on 10 l of culture medium; (2) the influence of the physiological state of the inoculum.

An accurate method for the determination of 3-ketoglycosides was needed in order to follow their formation. It was shown previously that the latter compounds react with both *o*-phenylenediamine-HCl at 100°C. and with semicarbazide at 30°C. and that they form derivatives with absorption maxima in the ultra-violet range. These two reactions have been compared as quantitative analytical methods. The second reaction is more specific, permitting a more accurate determination of the 3-keto-compounds.

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[1]) Geassocieerde Nationaal Fonds voor Wetenschappelijk Onderzoek.

METHODS.

Aeration of the culture medium: For laboratory scale production of 3-ketoglycosides a reciprocal shaker was used making eighty 5 cm strokes per min. Round flat Fernbach flasks of 1 l total capacity were used, containing not more than 150 ml culture medium. The composition of the latter culture medium and its preparation were described previously (Bernaerts and De Ley, 1960). For larger scale production a fermentor was designed (see fig. 1) consisting of a cylindrical 20 l pyrex container of 30 cm diameter and 30 cm height and a fitting stainless steel cover with a rubber seal. Through the cover several stainless steel tubes were soldered, one of them (A) allowing to maintain a constant temperature in the culture medium by circulating a current of water at 35°C. through a spiral at the bottom of the container.

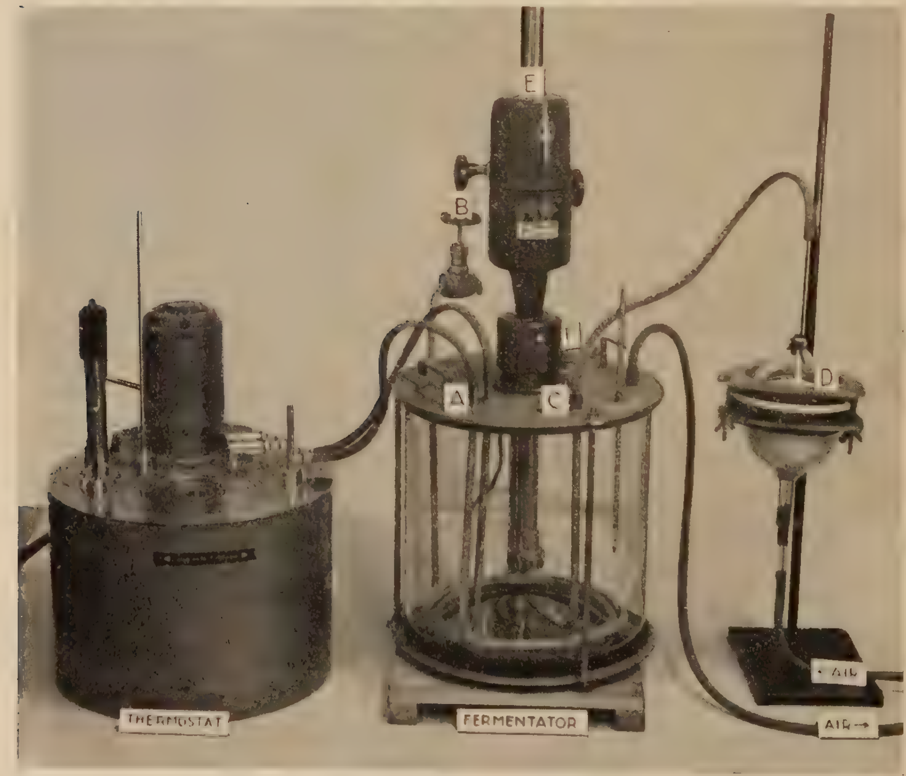

Fig. 1. Fermentor with thermostat and air sterilizer (for description, see text).

A second tube descended to the bottom of the container and was provided on the outside with a stainless-steel tap (B). Through it, samples were collected during the operation by effecting an over-pressure in the container, which forced the liquid into the opened tube. A third tube (C) led a current of air or oxygen, passed over sterile cotton (D), into the center at the bottom of the container. Efficient aeration of the culture medium depends upon bringing the liquid into intimate contact with the largest possible surface of air. [For a review of aeration problems, see CHAIN *et al.* (1952)]. A very efficient dispersion of the air or oxygen bubbles was obtained by means of an Ultra-turax, TP 45/6 mechanical stirrer [1]) (E) consisting of a circular comb rotating at high speed within a second fixed one. The shaft, bearing the rotating part, passed through the cover down to the centre of the liquid. Although the maximum speed obtainable was about 10,000 r.p.m., we used in all our experiments about 4,500 r.p.m. in order to avoid possible mechanical destruction of the organisms.

Quantitative measurement of the aeration rate was performed by the sulfite oxidation procedure of COOPER *et al.* (1944), modified by CORMAN *et al.* (1957). The oxygen absorption rate (O.A.R.) is defined as millimoles of oxygen absorbed per liter of solution per minute.

A large hole (F) provided with a screw stopper, allowed the addition of antifoam, $CaCO_3$ and ethylene oxide.

Excessive foam formation was avoided by the addition to the culture medium of about 200 p.p.m. Silicone M.S. antifoam "A" [2]).

The fermentor was sterilised inside with ethylene oxide. For that purpose a test tube, suspended from a wire and containing a few ml ethylene oxide was introduced into the fermentor through F and all the apertures were closed. 24 hrs later the test tube was removed and the ethylene oxide vapours eliminated by a stream of sterilized air or oxygen.

Afterwards 10 l of culture medium, sterilized separately, were introduced asepticaly into the fermentor and inoculated.

Determination of 3-ketoglycosides was carried out as described under "Results".

Preparation of the cell suspensions for inoculation. Bacterial cells, non-adapted to the disaccharide, were obtained from Roux flasks containing a culture medium with (% w/v) : 0.2 $(NH_4)_2SO_4$; 0.1 KH_2PO_4; 0.025 $MgSO_4 . 7\ H_2O$; 0.175 yeast extract (Difco); 2 agar and 1 glucose. Final pH 6.8. After 24 hrs incubation the young organisms were washed from the surface with physiological saline and harvested.

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[1]) Janke & Kunkel, Staufen, Germany.

[2]) Hopkins & Williams Ltd., Chadwell Heath, Essex.

Samples of chromatographically pure 3-ketoglycosides were prepared as previously described (Bernaerts and De Ley, 1960). No crystalline products have so far been obtained except for 3-ketolactose. The preparation of this pure compound is described under "Results".

RESULTS.

Determination of 3-ketoglycosides: 3-Ketoglycosides react with *o*-phenylenediamine-HCl at 100°C. and yield derivatives with an absorption maximum at 350 mμ (3-keto-lacto- and maltobionate) and at 335 mμ (3-keto-lactose -maltose and -saccharose). Unfortunately, this reaction is of little use for the estimation of 3-keto-compounds during the aerobic fermentation, since the remaining disaccharides react as well. The optical densities of different reaction products are for 100 μg of the compounds: lactose, 0.142 at 335 mμ; 3-keto-lactose, 0.825 at 335 mμ; maltose 0.185 at 335 mμ; 3-keto-maltose, 0.800 at 335 mμ; 3-ketomaltobionate, 0.750 at 350 mμ; 3-ketolactobionate, 0.600 at 350 mμ and 3-ketosaccharose, 0.750 at 335 mμ.

Better results were obtained with the method of MacGee and Doudoroff (1954) for the estimation of total α-ketoacids with semicarbazide. A small sample (*e.g.* 2 ml) of the culture medium was centrifuged at 5,000 r.p.m. for 20 min. The clear to opalescent supernatant was diluted 1 : 50 to 1 : 200 according to the presumed concentration of the 3-keto-compound. The 1 ml samples of these dilutions, containing between 50 and 400 μg 3-ketoglycosides, were mixed with 1 ml of the reagent (1% semicarbazide-HCl and 1.5% sodium acetate trihydrate in water), incubated for 15 min. at 30°C., diluted to 5 ml with water and the absorption read in the Beckman spectrophotometer, model DU, at 230 mμ in a 1 cm quartz cuvette against a diluted reagent blank.

A reference curve obtained with pure crystalline 3-ketolactose is represented in fig. 2. Similar reference curves with chromatographically pure samples of other 3-ketoglycosides showed values which are 10 to 20% lower. Perhaps these differences are due to the lower purity of these last samples.

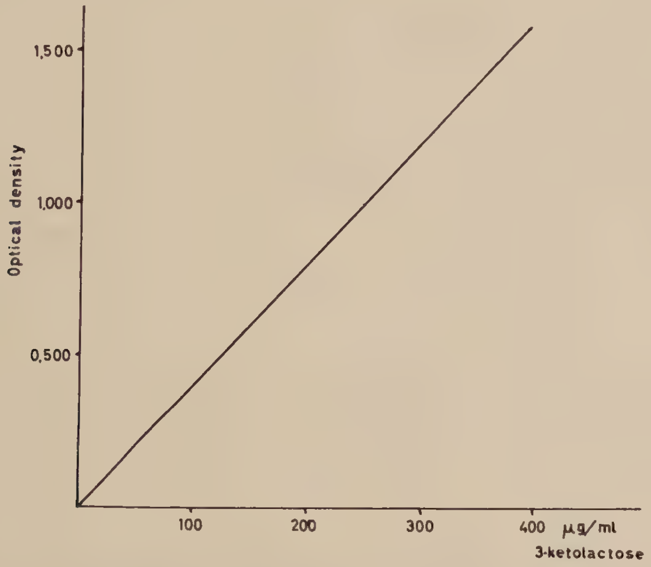

Fig. 2. Absorption values (optical density) at 230 mμ of the reaction product of 3-ketolactose with semicarbazide.

From the concentrations the yield of the 3-ketoglycosides during the aerobic fermentation process could be calculated. The latter method was preferred over the *o*-phenylenediamine procedure, because neither lactose, maltose nor impurities from the culture medium appeared to interfere.

Influence of inoculation with adapted and non-adapted organisms.

The amount of 3-ketoglycosides in aerobic fermentation processes increased during the first few days to reach a maximum and then slowly decreased. In order to obtain the highest possible yield it was important to get this maximum as soon as possible. We frequently observed that, if a good yield was not obtained during the first 24 to 36 hrs, *e.g.* by interrupting the aeration for a few hours, it never reached its optimal value. This was particularly true for substrates such as maltose, which usually gave low yields.

A fast production was obtained by using heavy inoculations with young organisms. As pointed out previously (Bernaerts and De Ley, 1960) several mechanisms seemed to compete for the catabolism of disaccharides: oxidation at C_3, hydrolysis and possibly polysaccharide production.

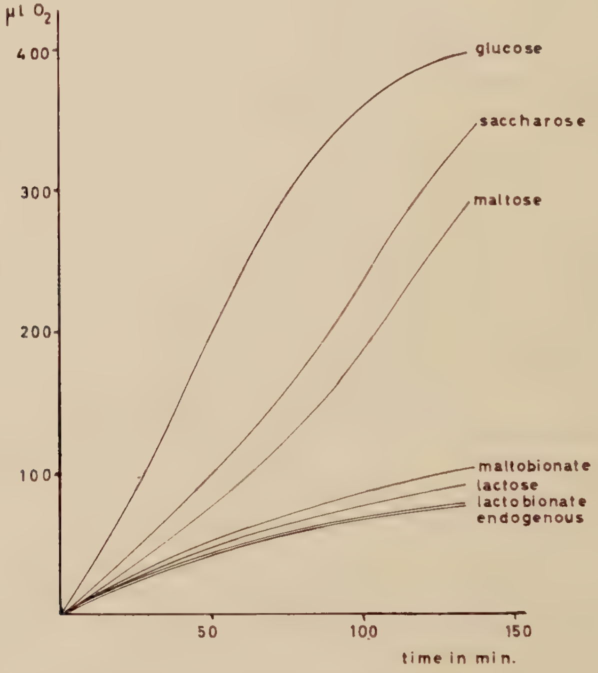

Fig. 3. Oxidation of several substrates by resting cells of the active strain, grown on glucose.

The hydrolases, responsable for the splitting of the oxygen bridge were adaptive enzymes as can be seen from fig. 3. Therefore it was expected that a heavy inoculation with non-adapted organisms would initially favour the oxidation at C_3 at the expense of the hydrolytic pathway. This hypothesis was repeatedly confirmed, an example being given in Table 1. This series of experiments concerned three different levels of inoculation with organisms cultivated on either a saccharose or a glucose medium in Roux flasks as described above. The aerobic fermentations were performed in Fernbach flasks on a shaking device. After 16 and 24 hrs, samples were withdrawn from the flasks and the concentration of 3-ketoglycosides determined according the method of MacGee and Doudoroff.

TABLE 1.

Relationship between nature of inoculum and production of 3-ketoglycosides.

Number of organisms/ml	Concentration in % of 3-ketoglycosides obtained from 4 % saccharose with organisms			
	grown on saccharose		grown on glucose	
	after 16 hrs	after 24 hrs	after 16 hrs	after 24 hrs
24×10^{13}	—	0.10	0.39	0.81
12×10^{13}	—	—	0.20	0.66
4×10^{13}	—	—	0.09	0.36

Influence of the aeration rate. The degree of aeration of the culture medium appeared to be another important factor for the production of 3-ketoglycosides. Aeration rates as realised *e.g.* in Fernbach flasks on a reciprocal shaker, were not very efficient. Relatively low values of about 1 for the O.A.R. were obtained.

The latter culture method was useful when small amounts of 3-ketoglycosides were needed. For larger scale production the fermentor, as described above, was more convenient. It has been shown that the O.A.R. values obtained with this device depend on the rotating speed of the stirrer and on the rate of air or oxygen supply. In order to prevent as much as possible the mechanical destruction of living organisms the speed of the rotor was kept at 4,500 r.p.m. The O.A.R. values obtained at 20°C. in relation with the rate of air or oxygen passing through 10 l of sulfite solution are given in fig. 4.

High values for the aeration rate could easily be obtained by increasing the air supply. However a flow rate of 20 l of air/min was considered as a practical upper limit because with larger amounts excessive foam formation was difficult to avoid without the use of large amounts of antifoam. Very high values of the O.A.R. could be obtained with pure oxygen at a reasonable flow rate (see fig. 4).

Several experiments were performed using 10^{14} to 10^{15} non-adapted organisms/ml as inoculum and with varying aeration rate. Maltose and saccharose were selected as substrates in attempts to improve the yield of oxidation products because usually very low and variable yields were obtained in the Fernbach aeration

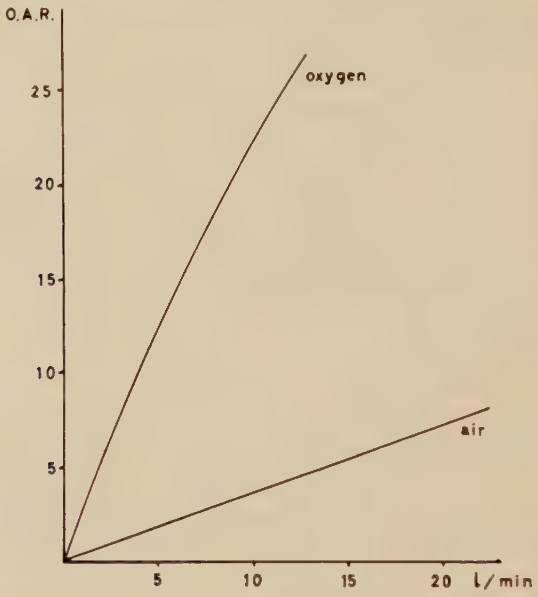

Fig. 4. Relation between the oxygen absorption rate (O.A.R.) and the supply of pure oxygen and air.

technique. With relative feeble aeration rates up to an O.A.R. value of about 4, the yield of oxidation products depends on the aeration rate. With O.A.R. values of 4 up to 15, no noticeable increase of the yield was obtained. For this reason an O.A.R. of 6, corresponding to a flow rate of 16 l air/min was ordinarily used. In these conditions we obtained the highest and the most reproducible yields of 3-ketoglycosides with either maltose and sucrose. About 12% of maltose and 20% of saccharose was converted into 3-ketoglycosides (initial concentration of disaccharides : 4%).

Other substrates such as lactose, lacto- and maltobionate were converted into the corresponding 3-keto-compounds with about the theoretically expected yield.

Influence of the composition of the culture medium. Several modifications in the composition of the culture medium such as the use of corn steep liquor, larger amounts of yeast extract, and varying concentrations of the substrate, were without effect or even produced a harmful influence on the yield of 3-ketoglycosides.

Preparation of pure crystalline 3-ketolactose. The culture, obtained after an aerobic fermentation of 10 l medium containing 4% lactose monohydrate, was centrifuged and the opalescent supernatant fluid was treated with equal volumes of 0.1 M $K_4Fe(CN)_6 . 3H_2O$ and 0.2 M $ZnSO_4 . 10\ H_2O$ until all protein was precipitated. The cations were absorbed by pouring the transparent yellow liquid successively on two strong cation exchanger (Merck I) columns (height 50 cm; diameter 4 cm). The acid solution was afterwards poured on two similar columns of weak anion exchangers (Merck II). The resulting colourless and odourless solution had a pH between 5 and 6 and was concentrated by large-scale lyophilisation. Paper chromatography of a 10% solution, using water-saturated phenol as a solvent, showed only traces of substrate and an intense spot of 3-ketolactose.

The pooled viscous concentrates were treated with an excess (5 l) of methanol and the resulting precipitate (apparently polysaccharides) was filtered off. The colourless and clear methanol solution was kept for several days in the refrigerator at —10°C. and a white crystalline deposit was formed at the bottom of the vessel. Several fractions were isolated from the mother liquor and chromatographically checked. The first fractions (about 120 g) contained no detectable amounts of lactose (less than 1%); the following fractions (110 g) contained small amounts of substrate.

Discussion.

From our previous investigations on the formation of 3-ketoglycosides we know that several mechanisms are involved in the catabolism of disaccharides by this strain. The hydrolytic pathway, the oxidative route and probably the formation of polysaccharides compete for the substrate. The most favorable conditions for the oxidative route, with the subsequent formation of 3-ketoglycosides have been investigated. The optimal conditions were hitherto not sufficiently clearly defined. With some substrates such as lactose, malto- and lacto-bionate these conditions are easily obtained. A good aeration of the culture medium and an inoculation with a heavy suspension of young organisms always guarantee good yields. For maltose and saccharose the use of organisms non-adapted to the substrate is indispensable. The oxidative route seems to be particularly important during the first hours of the culture process.

The non-adapted organisms are not capable to hydrolyze the disaccharide substrate in the early stage of the fermentation and it seems probable that the oxidative process gains in importance. It is therefore also important to start with the highest possible number of organisms in order to attack the substrate "en masse". It seemed interesting to try this oxidation with heavy concentrations of resting cells. The results were however very poor and only low yields of oxidation products were obtained from substrates which are otherwise easily oxidized, whereas maltose and saccharose were not converted.

Summary.

Improved conditions were described to increase the yield of 3-ketoglycosides, which are formed during the oxidation of several disaccharides and bionates by a strain of aerobic bacteria. These end-products were formed fastest when a heavy inoculum (10^{14}—10^{15} cells/ml) of non-adapted organisms was used. The aeration rate was found to be very important. An oxygen absorption rate (O.A.R.) of 6 was currently used. In these conditions a 4% solution of lactose, lacto- and maltobionate was nearly quantitatively converted into the corresponding 3-keto-compounds. Maltose and saccharose gave a yield of 12 and 20% respectively. An analytical method to determine 3-ketoglycosides with semicarbazide was described. 3-Ketolactose has been obtained in the crystalline state.

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NETHERLANDS SOCIETY OF MICROBIOLOGY MEETING ON APRIL 15, 1961

A STUDY OF THREE FACULTATIVELY ANAEROBIC CYTOPHAGA'S

by

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(Microbiological Laboratory, Wageningen, The Netherlands).

The organisms were isolated from marine mud by Dr. J. Lascelles. One organism which forms a pale yellow pigment when grown aerobically is considered to be similar to *C. fermentans* Bachmann (Bachmann, 1955). It will be described as a variety of *C. fermentans* because of its ability to digest agar.

The main difference between the two other isolates which form a red pigment, is their behaviour towards agar. One organism is able to grow at the expense of agar, whereas the other is not.

All organisms are characterized by flexing and gliding motility and by the absence of microcysts and fruiting bodies.

The organisms can be grown in a mineral glucose medium, although growth is accelerated and more abundant when a vitamin mixture is included. Carbon dioxide is an absolute requirement for growth.

The growth of the two agar decomposers is markedly inhibited when cultivated aerobically on plates containing 0.1% of Seitz-sterilized glucose or galactose.

Acetic, propionic, and succinic acids are major end-products of sugar fermentation by all three isolates. The red pigmented strains form in addition CO_2 and H_2, as well as small amounts of lactic and formic acids, and of ethanol.

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ANAEROBIC ALLANTOIN DEGRADATION

by

G. D. VOGELS

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Up till now *Streptococcus allantoicus* (Barker, 1943) is the only organism known to possess the capacity of growing anaerobically with allantoin as source of carbon, nitrogen and energy. From ditch mud we have isolated a *Streptococcus* strain and a gram- and catalase-positive rod, which both grow well in Barker's allantoin medium under anaerobic conditions.

In the fermentation so far two pathways could be distinguished: on the one hand a hydrolytic pathway resulting in the formation of urea, glyoxylic acid, glycine and cell material *via* allantoic acid as an intermediate; on the other hand, an oxidative pathway in which oxaluric and oxamic acids are formed *via* a dehydrogenation of allantoin. Urea was lacking among the end-products of fermentation by the rod; the role of this compound as an intermediate was evident from its accumulation during the degradation of allantoin in the presence of iodoacetate.

In the course of the fermentation by growing cells, cell suspensions and cell-free extracts of the two organisms appreciable amounts of allantoic acid could always be detected on the paper chromatograms of the incubated mixtures. Oxaluric acid accumulated when EDTA (30 mM) had been added to the reaction mixture. These two intermediates were identified by comparing their paper chromatographic behavior with that of authentic oxaluric and allantoic acids in four different solvents.

Oxaluric acid is broken down according to a reaction scheme also found by Valentine and Wolfe (1960): out of one mole of oxaluric acid, one mole quantities of ammonia, carbon dioxide and oxamic acid were formed respectively. On addition of ornithine, citrulline was produced, suggesting carbamyl phosphate as an intermediate.

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INCREASE OF *Proteus* INFECTIONS OF THE URINARY TRACT, PROBABLY CAUSED BY THE USE OF CHLORHEXIDINE IN UROLOGY

by

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(Binnengasthuis, Amsterdam, The Netherlands).

During the past four years we observed an increase of serious urinary tract infections, caused by *Proteus rettgeri*. In all patients catheterisation, cystoscopy or treatment with an indwelling catheter preceded the appearance of the infection. Before 1957 *Proteus rettgeri* was only isolated in very few instances in our laboratory but after 1957 it was isolated from the urine in 90 patients and from the blood in 2 patients.

Chlorhexidine was introduced in urological wards in 1957 and is now the only antiseptic used. On investigating the source of these infections it was found that the growth of *Proteus* is not inhibited by the concentration of chlorhexidine used in our urological ward in solutions for irrigation of the bladder, *i.e.* 1 : 5,000.

Of 58 strains, recently isolated from urine, the minimum bactericidal concentration of chlorhexidine-digluconate was determined. The results are presented in table 1. Inoculation was made with a 1 : 1,000 dilution of an 18 hrs broth culture; inoculation with a 1 : 100 or 1 : 10 dilution caused already growth in a concentration of chlorhexidine twice or four times higher than the results indicated in the table.

TABLE 1.

Sensitivity of various bacteria towards chlorhexidine.

Organism	Number of strains	Minimum bactericidal concentration of chlorhexidine after 48 hours at 37° C.							
		1: 2,000	1: 4,000	1: 8,000	1: 16,000	1: 32,000	1: 64,000	1: 128,000	1: 256,000
Proteus rettgeri	15	1	3	7	4				
Proteus mirabilis	16	2	9	5					
Proteus vulgaris	4		2	1				1[1]	
Ps. pyocyanea	8				2	6			
E. coli	7						1	3	3
Aerobacter	2					2			
Staph. albus	2								2
Staph. aureus	2								2
Strept. faecalis	2						2		

[1] This was the only strain not recently isolated from urine. It was a non-swarming strain, maintained for years in the laboratory.

ANTILEUCOCIDIN AND RESISTANCE AGAINST STAPHYLOCOCCAL INFECTIONS

by

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(Laboratory of Microbiology, Utrecht, The Netherlands).

Several staphylococcal toxins are injurious to red or white blood cells. They can be distinguished by the specificity with regard to species and cell type. The α-hemolysin damages the red cells of various species and is toxic for rabbit leucocytes, but not for human leucocytes. The Panton-Valentine leucocidin is not hemolytic. It damages and eventually kills human leucocytes and the white cells of the rabbit but is not injurious to leucocytes of guinea pig, mouse and frog.

The damage caused by the Panton-Valentine leucocidin shows a specific pattern, which can be followed by phase-contrast microscope. The cell takes a spherical form, the nucleus loses its segmental structure, becomes pycnotic and spherical and takes a central position. The granules migrate towards the cell-wall (GLADSTONE and VAN HEYNINGEN, 1957). These changes can be used for an estimation of this toxin. One MLeD (Minimum leucocidal dose) was defined as the concentration which produces the described morphological changes.

As the leucocidin is antigenic, antibodies can be obtained and titrated by a neutralisation test. One anti-leucocidin unit was defined as the quantity neutralising 500 MLeD. Though this method is cumbersome it is as yet the most specific.

Anti-leucocidin titers were determined in the peripheral blood of 234 women immediately post partum and again after a period of 2 months.

The frequency percentage of the initial titers are shown in the first histogram (fig. 1*a*). For comparison the anti α-toxin titers are given in fig. 2*a*. During the observation period of two months a number of clear-cut cases of puerperal mastitis was observed; other staphylococcal infections were too rare to permit conclusions. The distribution of the cases of mastitis over the groups with different titers of both antitoxins are plotted (in absolute numbers) in fig. 1*b* and 2*b*. It is obvious that cases of mastitis occur only in women with low titers of anti-leucocidin.

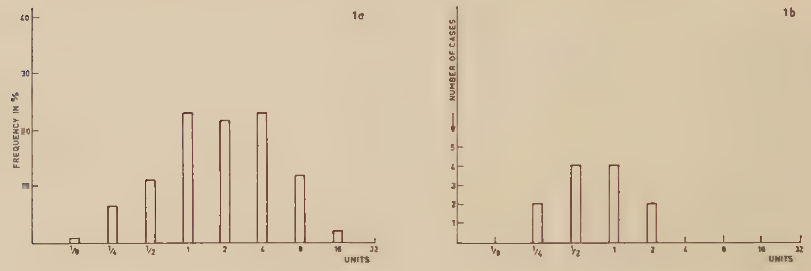

Fig. 1.

a. Frequency distribution of anti-leucocidin titers in anti-leucocidin units of 234 lying-in women.

b. Absolute numbers of cases of puerperal mastitis in each group.

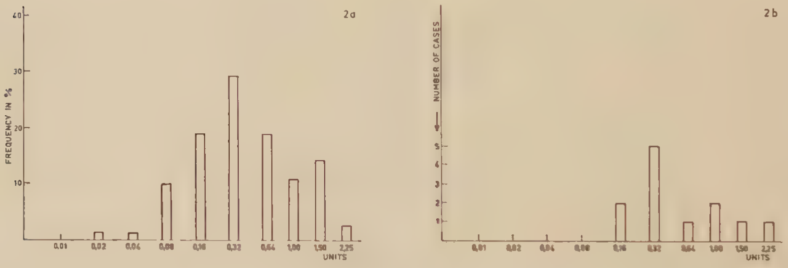

Fig. 2.

a. Frequency distribution of anti α-toxin titers in antitoxin units of 234 lying-in women.

b. Absolute numbers of cases of puerperal mastitis in each group.

No such correlation with anti α-toxin is observed. The 234 subjects can be divided in 150 mothers with titers ≤ 2 units of anti-leucocidin and 84 with titers above 2. All 12 cases of mastitis fall in the first group (P 0.02).

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PRELIMINARY REPORT ON MORPHOLOGICAL STUDIES OF "L" COLONIES OF TWO DIFFERENT SPECIES OF BACTERIA

by

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(Municipal Bacteriological Laboratories, Ziekenhuis Dijkzigt, Rotterdam, The Netherlands).

Considering our lack of knowledge of the existence of "L" organisms in nature a morphological study is thought to be the most promising for the present.

The transformation of the staphylococcus into the "L" phase was traced using time lapse photography (Schönfeld, 1961). Intermediate stages between the bacterial and the "L" phase were not found. The reversion from the "L" phase to the coccus was too freakish to enable us to try serial photography. "L" forms of staphylococcus have been subcultured in liquid medium and freeze-dried.

In cultures of mycobacteria (H 37, BCG, Johne's bacillus), using three different media, tiny colonies — 10-15μ in diameter — were observed. They are greatly similar in structure to the streptobacillus "L" forms (cf. Klieneberger, 1960), but slightly different from small "L" colonies of staphylococcus. They have been subcultured on solid medium but the number of colonies on 1 cm^2 agarblocks used to be very small. Recently the subculture into liquid medium was found to be possible. The realisation of serial photography must await better understanding of the conditions favourable for the transformation. It was not yet possible to make these "L" colonies revert into the mycobacteria.

When speculating on the existence of the organisms *in vivo* three characteristics should be taken into account:

1. the resistance to antibacterial substances and to antibodies.
2. the lack of pathogenicity to human and animal tissues.
3. the freakish reversibility.

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PROTECTIVE EFFECT OF INTERFERON IN INFLUENZA-INFECTED MICE

by

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Interferon was discovered by Isaacs and Lindemann in 1957 while studying virus interference. These authors have shown that this compound, probably a protein, is formed by the interaction of influenza virus and the host cell. Interferon is able to inhibit the growth of a number of viruses *in vitro*. Some evidence has been supplied to demonstrate that interferon could also be active *in vivo*, namely in mice (HITCHCOCK and ISAACS, 1960). Interferon could therefore represent a new approach to the control of virus infections.

In investigating such a possibility, knowing that interferon, although cell specific, is not virus specific, we decided to induce the production in the host itself rather than to administrate interferon prepared *in vitro*. In our experiments, groups of mice were treated by intra-nasal inoculations of UV-irradiated influenza virus strain A.PR$_8$ or A.Sing. At intervals, these animals were challenged with a LD$_{50}$ dose of live influenza virus strain A.PR$_8$ or B.Lee. In most experiments we used a mouse-adapted strain of influenza virus.

Groups of control mice received only the challenge. Replicate experiments showed that the percentage of survival in the pretreated mice was close to 100% whereas survival in the control groups was only 30 to 50%. In studying this phenomenon by challenging at different periods of time, we observed that protection appeared quite rapidly, around 24 to 48 hrs, and disappeared abruptly around the 5th or 6th day after pretreatment. This effect could not be attributed to the presence of antibodies. Antibodies did not appear before the 8th or the 10th day, which was too late to affect the course of infection. Moreover in some experiments, the challenge virus was heterotypic.

From these data, we conclude that interferon can be induced in the host and that it is able to protect mice efficiently against influenza infection. Such a finding could lead to the application of interferon in the prophylaxis of human viral respiratory infections. Experiments are now in progress to establish this fact.

HITCHCOCK, G. and ISAACS, A. 1960. Brit. Med. J. Nr. 5208, p. 1268.
ISAACS, A. and LINDEMANN, X. 1957. Proc. Roy. Soc. 147, 258.

ENERGY PRODUCTION IN *Gluconobacter liquefaciens*

by

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Gluconobacter liquefaciens is a strictly aerobic organism in which glycolysis is absent and which oxidises all substrates *via* the pentose phosphate or the citric acid cycle.

Consequently energy can only be obtained from phosphorylation in the respiratory chain. The extent of this oxidative phosphorylation has been measured in cell-free extracts in a system analogous to that of BRODIE (1959). The P/O ratios found for several substrates are: glucose 0.18 (oxidation to gluconate); ethanol 0.09; DPNH 0.09; TPNH 0.22; citrate 0.22; succinate 0.16; α-ketoglutarate 0.22; lactate 0.13; glycerol 0 and G-6-P 0.40. For G-6-P this would mean about 5 moles ATP per mole G-6-P on the assumption of complete recycling in the pentose phosphate cycle. The P/O ratios are low in comparison with those found for mitochondria (DPNH P/O 3.0), but similar to those found with other bacteria. During substrate oxidation by resting cells, ca. 40% of the substrate is assimilated. From this the ATP yield/mole substrate oxidized can be estimated by assuming that 1 mole substrate is assimilated at the cost of 3 ATP (as for glycogen formation). By determining the cell yield per mole substrate in growing cultures it is also possible to calculate the gain of ATP per mole substrate. For this estimation we used the figure of Bauchop and Elsden (1960), who found with 3 different micro-organisms, that the growth yield was approximately 10.5 mg per mole ATP. In synthetic medium 1 mole galactose gave a growth yield of 20.5 mg cells; 1 mole fructose one of 19.4 mg cells.

With these 3 methods comparable results were obtained. About 3 moles ATP are formed by the complete oxidation of 1 mole galactose, glucose or fructose. This means that the free energy of oxidation is used very inefficiently in this organism.

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Bauchop, T. and Elsden, S. R. 1960. J. Gen. Microbiol. 23, 457.

MECHANISM OF ACTION OF INTERFERON

by

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Several workers have described the inhibition of virus growth within infected cells treated with interferon. It has been shown that interferon has no effect on extracellular virus and that it does not affect virus absorption into the host cell. These results suggest that the mechanism of action of interferon is intracellular.

Isaacs *et al.* (1961) have shown biochemical and biological similarities of behavior between interferon and known substances which uncouple oxidative phosphorylation. These authors think that this might represent the mechanism through which interferon inhibits virus synthesis in the cell. Several findings support their hypothesis.

In the course of our investigations about interferon influence on isolated polio RNA, using either tissue culture system or embryonated eggs, we have observed that mixing *in vitro* of polio RNA and interferon or simultaneous inoculation of both compounds results in a complete inhibition of viral synthesis. It has been shown that infection of the embryonated egg with polio RNA results in a one cycle multiplication of virus. From these observations, we have developed the hypothesis that interferon might act by a direct interaction with infecting viral RNA. We have also studied this phenomenon in cells infected with WEE virus. When infectious RNA is extracted from cells at early periods after infection, it is possible to demonstrate with this virus the presence of a precursor RNA or of true viral RNA, depending on the temperature of extraction. With this method, we have shown that interferon action was already demonstrable at the time when only precursor RNA is present in the cell. This would suggest that blocking of RNA formation by interferon occurs at an early phase in viral infection.

To study further this hypothesis we have used a methylated protein as a model binding agent. This protein has been described as a specific adsorbant for nucleic acids on column chromatography. *In vitro* combination of viral RNA and methylated protein results in a loss of RNA infectivity and a protection from RNAase action. The association is reversible and infectious RNA can be liberated. The similarity of action between this known binding agent and interferon supports the hypothesis that interferon could act by its binding capacity for RNA. Interferon represents a specific protein, the presence of which is induced in the cell by viral information at the same time as the development of viral infection. The inhibitory action of interferon might represent a spontaneous control of this infection by interaction with viral RNA. In this way, interferon action could be integrated in a more general pattern of nucleic acid metabolism, similar inhibitory systems explaining the control of cellular RNA in the normal cell.

Isaacs, A. *et al.* 1961. Virology **13**, 191.

EGG MEDIA FOR THE CULTIVATION OF MYCOBACTERIA

by

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Basing his investigations on findings with the Pyruvate medium (1951, 1958), the author developed a new glycerol egg medium which may be used as a substitute for the Loewenstein medium (1930). In this investigation the 10 colony method was used, which is based on the linear relations between the logarithm of the number of colonies, the logarithm of the dilution and the time of first appearance of colonies. This method gives quantitative comparisons of "velocity" and sensitivity of different media, standardized on the same amount of culturable bacterial units. The medium has received the name of Glycerol 6 and consists of a phosphate buffer with pH 6.5, glycerol egg mixture and malachite green. The properties are the same as those of the Loewenstein medium: good growth of normal human, avian, atypical and saprophytic strains; restricted (dysgonic) growth of bovine, murine and isoniazid resistant human strains; good formation of pigment of chromogenic strains; results of catalase and niacin tests similar to Loewenstein medium. In sensitivity tests results could often be read earlier on the Glycerol 6 than on Loewenstein medium. Readings of streptomycin sensitivity were generally slightly higher on Glycerol 6.

In contrast to Glycerol 6 medium, growth of bovine, murine, and isoniazid resistant human strains was eugonic on Pyruvate medium. This medium, however, was not suitable for sensitivity tests for isoniazid, since pyruvate and isoniazid are antagonists.

Normal human strains grew more rapidly on the Pyruvate medium than on the other two media. The colony morphology of true tubercle bacteria on Pyruvate medium differed markedly from that of avian, atypical and saprophytic Mycobacteria.

In first isolations, performed simultaneously on Pyruvate and Loewenstein media, the former was definitely superior. All strains with a resistance of 10 microgram isoniazid in 1 ml of medium grew better on the Pyruvate medium.

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CONVERSION *IN VITRO* OF A HUMAN PLEUROPNEUMONIA-LIKE ORGANISM TO A *CORYNEBACTERIUM*[1]

by

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(Received March 6, 1961).

INTRODUCTION.

The designation of a pleuropneumonia-like organism (PPLO) as a member of the order *Mycoplasmatales* (EDWARD and FREUNDT, 1956) depends ultimately upon its lack of demonstrable identity with any member of another bacterial order.

Several workers have reported the derivation of typical bacteria from cultures resembling PPLO. PPLO of avian origin were studied under a variety of conditions and were reported to give rise to gram-positive cocci or to pleomorphic rods (KELTON and GENTRY, 1957; GENTRY, 1960; KELTON, GENTRY and LUDWIG, 1960). McKAY and his associates also studied avian PPLO and claimed that *Hemophilus gallinarum* evolved from them during passage in eggs (McKAY and TAYLOR, 1954; McKAY and TRUSCOTT, 1960). Organisms resembling PPLO have been isolated repeatedly from the human genital tract, and such isolates have occasionally been reported to give rise to bacteria (Tulasne, 1951; Minck, 1953a, 1953b; Moustardier *et al.*, 1953). A human urethral PPLO (strain Campo) was studied extensively by Smith and his coworkers and was reported to give rise to diphtheroids on numerous occasions (Peoples, Smith and Morton, 1955; Smith, Peoples and Morton, 1957; Smith and Rothblat, 1960). Another human genital strain gave rise to *Corynebacterium* during passage in HeLa cell cultures in the hands of Wittler *et al.* (1956). These and other reported reversions of PPLO to bacteria prompted examination of the ability of one typical human genital PPLO to revert to bacterial form under *in vitro* conditions.

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[2] This paper is part of a dissertation presented to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph. D. degree in Microbiology. Mailing address: Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D. C.

MATERIALS AND METHODS.

Organism. The pleuropneumonia-like organism (PPLO 4387) used in these studies was originally isolated from the urethral exudate of a patient with non-gonococcal urethritis. It was the strain reported by Wittler *et al.* (1956) to have reverted to a *Corynebacterium* during passage in HeLa cells in the presence of yeast extract. Criteria were given in that report for its designation as a typical human genital PPLO and for its differentiation from typical cultures of bacterial L forms. The strain was repeatedly repurified since then by transfer of agar blocks bearing single colonies, and no signs of a mixed culture were detected during more than 4 years of constant study. Furthermore, a culture of this organism was sent to Dr. Emmy Klieneberger-Nobel at the Lister Institute, London, England for comparison with human PPLO strains in her collection. On the basis of its cultural and morphological characteristics, Dr. Klieneberger-Nobel found this strain typical of *Mycoplasma hominis* and confirmed this identification by serological comparison with other strains of this species (personal communication).

Frozen cultures of the PPLO were thawed and maintained for the present experiments in flasks of veal infusion broth enriched with 25% ascitic fluid. Transfers were made at 2—3 day intervals and parallel agar cultures were examined microscopically at each transfer for evidence of atypical PPLO growth. Plate cultures of PPLO were inoculated by spreading a drop of a liquid culture over the agar surface with a glass spreader, and colonies were examined *in situ* on agar blocks by phase contrast microscopy and by the methylene blue-azure staining procedure of Dienes (Dienes, 1939; MADOFF, 1960). When signs of abnormal growth were detected in the passage stocks, they were replaced by fresh, typical cultures from the frozen stocks. Atypical cultures were never knowingly employed in reversion experiments.

Corynebacterium, C4387, was the strain isolated from the same urethral exudate from which the PPLO was isolated and was described previously (WITTLER *et al.*, 1956). The bacterium grew very poorly on routine media and somewhat better in the presence of 20—25% ascitic fluid. Good growth was obtained in the presence of 5—15% rabbit serum. Routine laboratory passage was in flasks of veal infusion broth enriched with 15% rabbit serum. Control platings on both ascitic fluid- and rabbit serum-veal infusion agar were routinely performed. After 24 hr. of incubation at 37° C., the *Corynebacterium* formed tiny but distinctly bacterial colonies on ascitic fluid and larger colonies (1—3 mm) on rabbit serum agar plates. Comparison of growth on the 2 media provided a check both on homogeneity of the stock and stability of its cultural characteristics.

Media. Routine media were prepared from Bacto veal infusion broth (VIB)[1]) and Bacto veal infusion agar (VIA)[1]) and were enriched with a final concentration of 25% ascitic fluid (AF) or 10—15% rabbit serum (RS).

All ascitic fluid was obtained from a single patient. Pooled normal rabbit serum was obtained from Cappel Laboratories, West Chester, Pennsylvania. Ascitic fluid and rabbit serum were resterilized by Seitz filtration immediately before use.

A broth medium (PPLOB) was prepared from a suspension of Bacto PPLO agar[1]) from which the agar was removed before heating by filtration through glass wool. After autoclaving it was enriched with AF or RS and then incubated for 4—5 days. The faint opalescence which developed from precipitation of constituents of the serum or ascitic fluid was removed by Seitz filtration before use.

A modification of the liquid medium of GILMORE and BURNETT (1959) was used in some of the reversion experiments. This was prepared as follows: 3.5 ml amounts of Bacto veal infusion broth (pH 7.4) were autoclaved in screw capped tubes. At the same time BBL fluid thioglycollate medium (pH 7.1)[2]) was sterilized in 10—15 ml quantities in individual large screw capped tubes. The following day a sterile 5 ml pipette was filled from the bottom of a tube of thioglycollate medium, inserted all the way to the bottom of a tube of veal infusion broth, and 3.5 ml delivered slowly under the veal broth. This resulted in a 1 : 1 ratio of thioglycollate medium to veal infusion broth. Ascitic fluid was added by running 2.5 ml from a syringe and needle down the wall of the tube. Even after 48 hr. of incubation without agitation, it was usually possible to detect visible boundaries between the zones of ascitic fluid, veal infusion broth, and thioglycollate medium. Tubes prepared in this manner will be designated V-T-AF.

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[1]) Difco Laboratories, Detroit, Michigan.

[2]) Baltimore Biological Laboratory, Inc., Baltimore, Maryland.

None of the media contained antibiotics, dye inhibitors, or thallium acetate. All media were incubated at 37° C. for 2—3 days before use and carefully examined for signs of bacterial contamination. For comparative studies, all media were prepared from the same stock bottles.

Corynebacterium filtrates. *Corynebacterium*, C4387, was grown on ascitic fluid- or rabbit serum-veal infusion agar (VIA-AF or VIA-RS) in Kolle flasks. The crops were checked microscopically for homogeneity and disrupted by oscillation in a 10 kc Raytheon sonic oscillator. Details of the methods employed were reported by MORRISON (1961). Sonic lysates were sterilized by membrane filtration through "Millipore Filters"[1]) with specified pore diameters of 0.45 μ, 0.3 μ or 0.1 μ.

RESULTS.

The pleuropneumonia-like organism, PPLO 4387, was previously reported to give rise to bacteria of the genus *Corynebacterium* during passage in tissue culture. Experiments were undertaken, therefore, to determine whether or not a similar reversion could be obtained in cell-free systems. Two distinct types of experimental procedures resulted in observable enhancement of the reversion phenomenon.

Effects of corynebacterial extracts on reversion. The rationale for the first series of experiments was suggested by the finding (LEDERBERG and ST. CLAIR, 1958) that deficiencies in metabolic systems leading to cell wall formation had an apparent effect on the development of L forms. The present studies were designed to determine the effect of adding constituents of the complete bacterial form to organism growing in the PPLO stage. The presence of extracts of *Corynebacterium* in growing PPLO cultures might supply missing cell wall intermediates and permit formation of complete bacteria.

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[1]) Millipore Filter Corporation, Bedford, Massachusetts.

Fifteen experiments were performed using essentially the same procedure in each. Cell-free filtrates of sonic extracts of the corynebacteria were prepared on the day of the experiment. Flasks containing 60 ml of ascitic fluid- or rabbit serum-veal infusion broth (VIB-AF or VIB-RS) were inoculated with 3 ml of a 48 hr. stock culture of PPLO and 1—3 ml of filtrate. Each experiment contained 4 experimental flasks and 8 or more control flasks containing either PPLO alone or filtrate alone.

Strict selective procedures were used in analyzing the results to eliminate experiments in which contamination or uncontrolled factors were involved. Criteria for rejection of an experiment included contamination in any flasks, evidence of viable forms in the filtrate by growth of corynebacteria in filtrate controls, and evidence of abnormal growth of the PPLO stock culture. Eight of 15 experiments were rejected by these criteria. The results of the remaining 7 experiments appear in table 1.

TABLE 1.

Reversion of PPLO to corynebacteria in the presence of sterile membrane-filtered corynebacterial extracts [1]).

Expt. No.	Numbers of flasks						Comments
	Experimental		Filtrate only		PPLO only		
	Total	Revers.	Total	Bacteria	Total	Bacteria	
3	4	2	4	0	4	0	
4	4	0	4	0	4	0	
5	4	1	4	0	8	0	
8	4	0	4	0	8	0	
9	4	1	4	0	8	0	
14	4	0	4	0	4	0	
15	4	0	2	0	6	0	[2])
Totals	28	4	26	0	42	0	

[1]) PPLO grown in ascitic fluid-veal infusion broth (60 ml) in the presence of 1—4 ml corynebacterial sonic lysate sterilized by "Millipore" filtration (indicated pore diameters: 0.45 μ, 0.3 μ, or 0.1 μ).

[2]) Extract possibly damaged by heating during sonic oscillation.

Of 28 experimental flasks containing both the PPLO culture and the filtrate, 4 (14.2 %) contained corynebacteria after 3 to 6 days. There were no indications of spontaneous reversion in any of 42 control flasks containing PPLO alone and no viable forms were detected in any of 26 flasks containing filtrate alone. In the flasks in which reversion did not take place, agar subcultures during the first week contained typical PPLO. Plates made during the second week contained decreasing numbers of typical PPLO colonies and increasing amounts of cellular debris and of degenerate and atypical colonies. Flasks were usually sterile after incubation for 2 weeks as shown by agar and broth subculture.

Of the 8 rejected experiments not included in table 1, 4 contained at least one flask inoculated with filtrate only in which corynebacteria appeared. In some cases, bacterial growth was not grossly evident until after incubation for 4 or more days, suggesting that the filter-passing organisms were minute granules or other atypical forms rather than normal bacteria. In one other experiment the PPLO stock was unstable; in 2 experiments contaminants were introduced with the rabbit serum or ascitic fluid enrichment; and in another experiment the bacterial extract was definitely damaged during the sonic oscillation.

The results of the valid experiments (table 1) indicated that the corynebacterial cell filtrates exercised a promoting effect on the reversion of PPLO to a bacterial form. However, the incidence of such induced reversion was not sufficiently high to warrant the use of this procedure as an experimental system for study of the details of this phenomenon. Therefore, efforts were made to find a more reliable system for inducing reversions.

Reversions arising during serial transfers in different media. A series of studies was undertaken based on the assumptions that the PPLO might pass through several growth stages during reversion to the *Corynebacterium*, and that media requirements might differ for the various intermediate stages. In addition to veal infusion broth, 2 other media described above were employed: (1) a mixture of veal infusion broth and fluid thioglycollate medium (V-T-AF), and (2) fluid media prepared from PPLO agar base (PPLOB-AF and PPLOB-RS). Similar media were used by Gilmore and Burnett (1959) for initial isolation and propagation of oral L forms and during stabilization of corynebacterial L forms. Furthermore, Wittler *et al.* (1960) used these media during studies on the derivation of bacterial forms from minute granular organisms from human clinical specimens.

For the experiments in this series a standard procedure was devised and used throughout. Protocol 1 outlines the transfer series used in the standard system.

PROTOCOL 1.

Standard procedure used in experiments on reversions arising during serial transfers in different media[1].

Stock PPLO flask of VIB-AF 48 hr. incubation	1 ml →	*V-T-AF* tube of V-T-AF
V-T-AF 72 hr. incubation	10 ml →	*P1* flask of PPLOB-AF[2]
P1 48 hr. incubation	4 ml →	*P2* flask of PPLOB-AF (standard flask)
P1 and *P2* 10—14 days incubation	→	Discard

[1] See text (pp. 267—268) for composition of media.

[2] In some sets PPLOB-RS was substituted for PPLOB-AF throughout.

Modifications in media and in transfer intervals were used in some experiments. One ml of stock PPLO culture was inoculated into each of a series of tubes containing V-T-AF. After incubation at 37°C. for 72 hr. the entire contents of the tubes were poured into flasks of PPLOB-AF or PPLOB-RS. The flasks were again incubated at 37°C. for 48 hr. and 4 ml transferred to a second series of flasks containing the same broth medium. All cultures were incubated for a further 10—14 days. As before, platings to rabbit serum- and ascitic fluid-agar were made at the time of each transfer and at 2—3 day intervals thereafter. Incubation times in the V-T-AF tubes (3 days) and in the first PPLOB-AF flasks (P1) (2 days) were strictly followed.

Table 2 indicates the results of the experiments of this type. During the first 3 experiments, reversions appeared in 4 of 6 flasks (P2) prepared exactly as in the protocol and in only 1 of 22 other cultures in the same medium but transferred according to other time schedules. Subsequent experiments were designed to test the ability of other types of fluid media to promote reversions. However, much lower rates of reversion were observed in the latter both in the standard control series and in the experimental modifications. No significant enhancement of the reversion rate was observed when thioglycollate-containing medium (V-T-AF) was supplemented with 1, 5, or 10μM/ml of glutathione, cysteine or methionine.

TABLE 2.

Reversions of PPLO to corynebacteria during serial transfers in different media.

Expt. No.	Number of flasks				Remarks
	Standard flasks 1)		Non-stand. flasks 2)		
	Total	Positive for reversion	Total 3)	Positive for reversion	
1	2	2	8	0	
2	3	1	4	1	
3	1	1	13	0	
4	2	0	10	0	Two non-standard flasks frankly contaminated.
5	6	0	8	0	Stock PPLO culture reverted during exp.
6	5	0	7	2	
7	2	0	8	0	Stock PPLO culture atypical. No spontaneous reversion.
8	3	1	12	1	
9	3	0	5	0	Two non-standard flasks frankly contaminated.
Totals	27	5	75	4	

1) P2 flasks only. Media and transfer intervals exactly as in standard system.

2) All other inoculated flasks, including P1 of the standard system.

3) Experiments 3—7 include flasks containing non-standard broth media.

Appearance of reverting cultures. During the serial transfer experiments, it was possible for the first time to make some observations on the stages immediately preceding the development of the bacteria. About the third day after inoculation, a detectable difference could be observed between the P2 flasks which later developed bacteria and those P2 flasks which were not destined to revert. The former, when unshaken and viewed in strong oblique light, contained a sediment of barely visible granulation on the bottom of the flasks. This sediment became somewhat coarser and more readily discernible during the next 2 days and finally developed white colony-like flecks. On swirling the flasks, many of these flecks remained adherent to the bottom. Transfer at this stage into VIB-RS accelerated the development of the normal *Corynebacterium* but a light growth also appeared after 2 more days without transfer.

Using a phase contrast microscope with an oil immersion objective, direct observations were made on wet mounts of granular sediments and on agar blocks prepared by placing a drop of culture on the surface of a sterile agar plate, cutting out a section of the agar, and mounting it as for PPLO colonies. Such preparations from normal PPLO cultures contained a variety of forms including granules, a few "ring-forms", tiny beaded filaments and weakly refractile round bodies from 0.5 μ to several μ in diameter. Reverting cultures contained more of the round bodies, and these appeared more refractile by phase contrast. Groups of very refractile round or oblong bodies about 2—3 μ in diameter somewhat resembling yeast cells were occasionally present. These forms were markedly different from other types of round bodies in the preparations. They were not detected in control cultures and may have been the bacterial precursors.

Agar plates inoculated from reverting flasks were incubated 48 hr. and the cultures examined microscopically. Agar subcultures made from young cultures in PPLOB-AF grew in the form of atypical PPLO colonies. Such colonies had very rough edges as opposed to the smooth round edges of typical PPLO, and the colonies lacked the granular core penetrating into the agar. The individual elements also varied much more than those of a typical PPLO colony and included granules, round bodies and large balloon-like vacuolar bodies. In these stages, the smallest granular forms were almost completely replaced by round bodies 1—3 μ in diameter. Agar subcultures, from slightly older PPLOB-AF cultures contained the same general type of colonies. However, some clusters of the round bodies appeared to be more refractile, and these clusters sometimes contained varying numbers of rod-like forms apparently growing, albeit slowly, as true bacteria.

Gram stained smears made from liquid cultures during the course of reversion were difficult to interpret. PPLO and transitional stages were gram-negative and usually consisted of faintly pink round forms similar to precipitated components of the medium.

In reverting cultures there often appeared masses of amorphous gram-negative matter, and the developing bacterial forms first appeared in these as tiny gram-positive specks and later as small groups of very small cocco-bacillary forms.

Observations on the PPLO stock cultures. The stock cultures of PPLO 4387 were routinely maintained in serial passage in flasks of VIB-AF. Observations were made on the stability of the cultures under these conditions. One stock line remained apparently unchanged for over 7 months in laboratory passage at 3—4 days intervals. During the eighth month of passage, the appearance of the colonies in the routine control agar platings became somewhat altered. The altered colonies had rougher edges than the colonies of previous typical passages, and granular elements had become larger and coarser. Spontaneous reversion to the corynebacterial form occurred about a month later in the serial broth passage of this line.

The effect of the transfer interval on the stability of the PPLO in routine passage was investigated. A fresh typical PPLO stock was divided into 3 parts which were then transferred at 1, 2 and 3 day intervals respectively. No spontaneous reversion appeared in the 1- or 2-day series during 7 weeks of cultivation. The organisms in these series sometimes formed an atypical rough type of colony but ordinarily returned to the normal form after 1—3 further transfers. In the 3-day interval series, the same cyclic pattern appeared, but a spontaneous reversion occurred in the flasks at the end of 3 weeks. A parallel series was carried on agar plates and transfers were made at 2-day intervals by pushing an agar block across the surface of a fresh plate. The PPLO carried in this way formed typical colonies throughout the experiment.

Studies on the cultural, morphological and biochemical characteristics of the derived bacteria. The criteria used for excluding contaminants have been indicated, and the stored bacterial cultures were considered to have arisen by reversion from a PPLO which in each case was a subculture of the same original strain. The bacteria arising from the PPLO were ordinarily transferred 1—4 times in rabbit serum broth and stored at —20°C. During these passages, considerable variation both culturally and microscopically was observed among the bacteria isolated from different experiments. This was an unexpected finding, and the frozen bacterial stocks were therefore re-examined and compared.

The derived bacteria all grew more luxuriantly on rabbit serum than on ascitic fluid media and grew sparsely or not at all on unenriched media. Good growth usually occurred in rabbit serum media within 48 hr. under aerobic conditions. Agar colonies were smooth, round, entire, raised and appeared moist and whitish. Most cultures produced colonies 1 mm in diameter after 24 hr. on the rabbit serum agar and smaller colonies (about 0.5 mm in diameter) on the ascitic fluid agar. One culture yielded colonies about 3 mm in diameter on VIA-RS. About 25 % of the cultures did not yield satisfactory growth on rabbit serum agar after 24 hr., and after 48—72 hr. gave very small colonies less than 0.5 mm in diameter. In 1 case, such colonies appeared initially as a bluish irridescence, and only with the aid of a hand lens could they be distinguished individually. Anaerobic conditions did not improve the growth of such cultures on agar. Prolonged serial passage was the best means found for improving their growth.

Gram stained smears of the cultures during the first 3—6 transfers after isolation from experimental flasks revealed organisms morphologically characteristic of corynebacteria, *i.e.* small, gram-positive, non-sporeforming rods. There was no question of mistaking even the shortest rods for cocci. Pairs of rods frequently occurred, but longer chains were never observed. Variations in the size, shape, and arrangement of the rods did occur. The most common form was a short, symmetrical rod about 0.7 μ in diameter and 1.5—2.0 μ in length with bluntly rounded ends. These rods occurred individually, in pairs, or in irregular clusters or palisades of 4—8 cells. Another form frequently observed was a rod of similar appearance and arrangement but much smaller, the rods often being less than 1 μ in length. In a few cases, organisms from early transfers took the gram stain very poorly. Smears from such cultures revealed gram-negative rods, round forms of various sizes, and amorphous matter interspersed with areas of minute, round, gram-positive granules mixed with tiny gram-positive rods. In addition, several cultures consisted of pleomorphic or club shaped gram-positive rods. Occasionally cultures contained larger, symmetrical rods with bands or bead-like inclusions.

Fifteen strains of reverted corynebacteria which had been stored in the frozen state from 1—15 months, were thawed and carried in parallel through 8—14 serial passages. During these passages, routine platings and gram stains revealed that the cultures were undergoing occasional random variations in colonial and microscopic morphology. The strains which had formed tiny colonies gradually became adapted to cultural conditions and grew almost as freely as the more "typical" bacterial isolates. Ten of the passaged cultures were inoculated in triplicate into semisolid fermentation media (BBL Trypticase agar base) enriched with rabbit serum. The carbohydrates added were dextrose, sucrose, maltose, lactose, galactose, rhamnose, dextrin, salicin or mannitol. Gelatin, nitrate media and litmus milk were simultaneously inoculated. The carbohydrate fermentation tubes were read at 2—4 day intervals for 3 weeks and acid production scored by color change of the phenol red in the media. No consistent fermentation patterns were observed among the strains with respect either to carbohydrates attacked or to the amount of acid produced. About 50 % of the cultures were weak fermenters producing only small amounts of acid from any substrate. The more vigorous acid-producers were most active in dextrose, maltose and dextrin. Two strains also formed acid from sucrose. Rhamnose and galactose were weakly attacked by most strains, while lactose, salicin and mannitol yielded essentially no acid with any strain. In some cases, differences appeared among triplicates identically inoculated into the same carbohydrate media. Three strains decolorized litmus milk, none reduced nitrate, and none liquified gelatin in 3 weeks.

It appeared that fermentation studies were not adequate to establish identity between the strains of derived bacteria. Therefore, studies were carried out to determine whether or not continuous fermentative and morphological variability was a characteristic of the bacterium.

From each of the 15 strains employed above, 4 cultures were selected which had been in storage for various periods under different conditions. All specimens were cultured in parallel under identical conditions yielding 60 sublines. Forty-four sublines (representing the 4 cultures derived from each of 11 of the original 15 strains) were morphologically indistinguishable. The remaining sublines were unstable and showed colonial or microscopic variations even among the 4 derived from a single strain and with sublines of different strains. The similarity among sublines of the 11 "typical" strains revealed in this experiment was much greater than the same strains had shown during the previous serial passage experiments.

A series of differential tests was carried out on the 11 strains which appeared to be comparable culturally and microscopically. For these studies a fluid fermentation medium was prepared (BBL trypticase, 1 %; NaCl, 0.5 %; rabbit serum, 3 %; sugar, 1 %; pH, 7.6) and sterilized by Seitz filtration. Carbohydrates used were dextrose, sucrose, and mannose. Triplicate tubes were inoculated and read at 3—4 day intervals. In this experiment more satisfactory consistency appeared among parallel tubes. However, as in the previous series which was carried out 10 weeks earlier using 7 of the same strains, no consistency was detected among the fermentative capacities of the different strains. Dextrose and mannose were strongly fermented by 4 strains, (*i.e.*, final pH below 7.0), less strongly by 5 (*i.e.*, pH between 7.1—7.3), and variably by 2 others, but these groupings were independent of the ability to attack sucrose. In all, 3 types of fermentative variation occurred: variation between parallel tubes, changes which appeared during the interval between the experiments, and inconsistencies between strains. Gelatinase activity was retested in triplicate tubes containing Bacto nutrient gelatin enriched with 3 % rabbit serum. After 13 days, 3 of 11 strains were positive for gelatin liquifaction.

Routine control platings prepared in parallel with the second series of fermentation studies again revealed colonial and morphologic similarity among the strains. However, in at least 4 strains the rabbit serum agar plates (VIA-RS) contained occasional lobulate colonies suggestive of papillae caused by variants arising during the course of colony development. A limited study was performed to test the possibility that such variation was responsible for inconsistencies observed. A fresh broth culture of one such strain was streaked on VIA-RS and incubated for 3 days at 37°C. followed by 3 days at room temperature. This treatment had previously been observed to promote the appearance of the lobulated colonies. A series of single colony isolations were made from this plate and were used to inoculate a fermentation series similar to the previous series. The sugars were dextrose, sucrose, maltose, mannose, and lactose. In this series, a characteristic fermentation pattern by most of the tubes of each set was evident. However, in at least 4 cases all of the parallel tubes (triplicates or quadruplicates) of a particular clone in a sugar varied from the normal fermentation type. These results tend to support the hypothesis that a high rate of variation in these corynebacterial cultures may account for the observed discrepancies in the biochemical characteristics of the different strains.

Discussion.

Evidence for reversion of a PPLO strain to a *Corynebacterium* depends on the validity of experiments showing that the reversion did not originate from contamination of the cultures, either from the laboratory environment or from materials added to the test systems.

Prior contamination of the medium from improper preparation of basal medium or from contamination of ascitic fluid or rabbit serum was controlled. All media were preincubated for 2—3 days before use to detect contamination, and each experiment included flasks of uninoculated media. All flasks were incubated about 2 weeks, providing sufficient time for such contaminants to manifest themselves. Two experiments in the first series were rejected because they contained contaminants presumably introduced by this route. In each case the contaminating organisms appeared early and at random in control and experimental flasks and could be easily distinguished microscopically from corynebacteria. The possibility that adventitious air borne contaminants may have entered during the experiments cannot be absolutely excluded. However, each experiment included at least twice as many control flasks as it did experimental flasks, and control and experimental flasks were handled in an identical manner. Among the experiments considered significant (table 1), no bacteria of any type ever appeared in 68 such controls, although some of the flasks were opened 4 or more times for control platings.

Another possible source of contamination was from viable forms in the filtrates prepared from the bacterial lysates. Corynebacteria occasionally appeared in such filtrates presumably because of flaws in the filter membranes caused by handling or excessive heat treatment. As controls to detect contamination from this source, all experiments included culture flasks inoculated only with filtrate in parallel with experimental flasks and also separate cultures of lysate on other media. The presence of bacteria of any kind in any control was considered sufficient reason for invalidating the experiment. In practice, however, all cases of contamination from this source were revealed by the presence of corynebacteria in all of the flasks inoculated with filtrate, although in a few cases they did not become evident until after incubation for 5—6 days. The rigorous criteria employed in selecting valid experiments permit a high degree of confidence that the results were not due to contamination.

That contamination was not responsible for reversions obtained during serial transfers in different media was supported by an additional factor. There appeared, in flasks where reversion was imminent, visible granulation which did not appear in control flasks. This transitional stage ordinarily persisted for 2—3 days and allowed some predictions as to which cultures were reverting. However in some cases where this type of granulation was observed, progression to the complete bacterial form failed to occur.

All experiments included controls to detect the presence of corynebacteria or other contaminants in the PPLO culture used as inoculum. These controls were considered adequate to detect either PPLO cultures in the process of spontaneous reversion or the presence of contaminants in the stock PPLO.

The low incidence (14 %) of reversion obtained by the addition of bacterial filtrates to PPLO cultures may have been due to suboptimal levels of some hypothetical reversion-promoting constituent of the filtrates. Rapid clogging of membrane filters and the necessity for extensive controls reduced the amount of filtrate added to each 60 ml of culture to less than 3 ml. Undetected variations in the state of the initial PPLO cultures may also have affected their ability to revert on certain occasions. Similar experiments have been reported by SMITH and ROTHBLAT (1960) during studies on the development of diphtheroids from the Campo strain of PPLO. However, they failed to detect an increased rate of reversion when the PPLO was cultured in the presence of a variety of cell wall constituents or diphtheroid fractions.

The experiments employing serial transfer through several media suggest the possibility that the initial stability of the PPLO culture is altered in some way by exposure to thioglycollate-containing medium. Subsequent incubation in other suitable media appeared to favor further progression toward forms similar to unstable L variants of bacteria. That these processes are not uniform was indicated by several observations. The occasional appearance of reversion in "non-standard" flasks with different transfer intervals suggests a variability in the rate at which the supposed "critical" state of instability was reached, and the frequent failure of reversion to appear in any flask suggests that the critical state may be transient or not attained in many cases.

The objective of this study was to demonstrate that a particular strain of PPLO, originally isolated from the human urethra and typical of *Mycoplasma hominis*, can give rise to a classical bacterial form and is therefore a variant growth form of the bacterium rather than a phylogenetically distinct type. Certain general conditions must be satisfied before this objective can be said to have been fulfilled. Among these are: (1) the PPLO strain must be a pure culture; (2) the derived bacteria must have originated from the PPLO beyond reasonable doubt, and (3) the derived bacteria must be the same in each case. The first condition, the purity of the PPLO, was satisfied by repurification procedures carried out both before and during the reversion experiments. Reasons have been given for believing that the second condition was satisfied by the experiments reported. Extensive studies have revealed marked similarities among the derived bacterial strains all of which are members of the genus *Corynebacterium*, but conclusive demonstration of their identity was not achieved as required by the third condition.

The initial bacterial isolates showed a wide range of variation both culturally and microscopically. This superficial variability was markedly reduced by prolonged serial passage and still further reduced after storage in the frozen or refrigerated states. However, a marked tendency to spontaneous variation was observed throughout the studies on the bacterial strains. If continuous variability was a characteristic of the bacterium itself, a rational explanation would be provided not only for the differences between strains but for the discrepancies among parallels. Fermentation tests were carried out on single colony clones from a single culture. Even in this experiment, discrepancies among parallel tubes were observed in a few cases. However, among sets where no such discrepancies appeared, at least 4 instances were observed where a clone fermented 1 of the 5 sugars differently from the other clones. While admittedly an inconclusive series, these observations support the view that continuous variations were occurring in the bacterial cultures and were responsible for the failure to demonstrate identity between them.

The results described here are compatible with the observations reported by Minck (1953a), Minck and Kirn (1960), and Peoples *et al.* (1955) all of whom obtained corynebacteria or diphtheroids from reverting human genital PPLO strains. The marked tendency of the reverting corynebacteria to vary spontaneously is in agreement with the findings of Wittler *et al.* (1960) on the variation of corynebacteria reverting from transitional forms. The tendency to vary has been reported for other types of bacteria obtained by reversion (Kelton *et al.*, 1960; McKay and Truscott, 1960).

The experiments reported in the present study have given persuasive evidence that at least one strain of human PPLO is capable of giving rise to a conventional bacterium. The existence of such evidence is itself a strong argument that this strain should be regarded as a variant growth form of the *Corynebacterium* sp. rather than a member of a taxonomically alien group, the *Mycoplasmatales*. More conclusive evidence of the relationship between the 2 types would require detailed information about the mechanisms of their interconversions and such information is not yet at hand. Two types of evidence are particularly desirable. These are (1) continuous morphological observations of the reversion of a PPLO to a bacterium and (2) derivation of a stable L variant from such a reverted bacterium followed by proof of its identity with the original PPLO. Taxonomic identification of human PPLO strains on a reliable basis can be expected only when their relationship to other bacteria has been satisfactorily described.

Summary.

Studies have been carried out on the derivation of a typical bacterium from a pleuropneumonia-like organism (PPLO) of human urethral origin. Wittler *et al.* (1956) had previously described the reversion of this PPLO to a *Corynebacterium* sp. during tissue culture passage. Two general procedures were developed which promote a low rate of reversion in cell-free media.

Reversion occurred in 4 of 28 trials in growth experiments when the PPLO was incubated in the presence of cell-free filtrates of the lysed *Corynebacterium* sp. Controls indicated that contamination of the filtrate or spontaneous reversion of the PPLO did not account for the appearance of the bacteria.

Transfers from a thioglycollate-containing broth to other broth media also resulted in reversions. When the latter broth was prepared from a dehydrated agar base, bacteria appeared by the third transfer in 5 of 27 trials using standard transfer intervals. With a variety of other media and transfer intervals, reversions appeared in 4 of 75 trials.

The derived bacteria were assigned to the genus *Corynebacterium* and all isolates displayed a number of basic similarities. Continuous variations in cultural and morphological characteristics were observed, however, and may be correlated with observed discrepancies in fermentation patterns among the isolates.

Strain PPLO 4387 was shown to revert to a typical bacterium *in vitro*, and the rate of reversion was increased experimentally. Possible implications of the experimental observations have been discussed, but a comprehensive description of the reversion process cannot be made at this time.

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CANDIDA INGENS NOV. SPEC.

by

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Although the elective medium for the isolation of *Brettanomyces* species is satisfactory for the rapid recovery of these yeasts from natural sources (VAN DER WALT and VAN KERKEN, 1961), it does not completely suppress the development of all other genera. Thus, an undescribed *Candida* species has been repeatedly recovered from such elective cultures. The new species, characterized by its unusually large cell-dimensions, appears to be widespread and has been recovered from material collected from a winery in the Western Cape Province, as well as from material from Bantu beer breweries and *Sorghum* malt in the Transvaal.

The following description of the species is based on the standard methods of WICKERHAM (1951, 1957) and LODDER and KREGER-VAN RIJ (1952).

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are round, oval, cylindrical, occasionally elongated (5—9) × (7 — 13 — 28) μ, single, in pairs, clusters or chains. A dull, dry, wrinkled, creeping pellicle and sediment are formed.

Growth on malt agar: After 1 month at 17°C., the streak culture is greyish-brown, dull, somewhat raised, flat or slightly wrinkled; the margin is lobiform, erose or filamentous.

Slide cultures: A primitive pseudomycelium is abundantly produced.

Fermentation: Absent.

Carbon assimilation: Glucose, galactose (latent), L-sorbose (latent), ethanol, glycerol, DL-lactic acid and succinic acid are assimilated but not maltose, saccharose, cellobiose, trehalose, lactose, melibiose, raffinose, melizitose, D-xylose, D-arabinose, L-arabinose, D-ribose, L-rhamnose, *i*-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, α-methyl-D-glucoside, salicin, potassium D-gluconate, citric acid and *i*-inositol.

Assimilation of ethylamine hydrochloride: Positive.

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Variable.

Growth in osmotic pressure medium: Absent.

Growth in the absence of an extraneous vitamin source: Positive.

Growth at 37°C.: Positive.

DISCUSSION.

Four strains of the species were recovered. These failed to sporulate as did all matings of these cultures. Since the species appears to be asporogenous and forms pseudomycelium, it is assigned to the genus *Candida*.

For the new species, the name *Candida ingens* is proposed in view of its unusual cell-dimensions.

Cultures of *Candida ingens* have been deposited in the yeast collection of the Centraal Bureau voor Schimmelcultures in Delft.

Candida ingens nov. spec.

In extracto malti cellulae rotundae, ovoidiae, cylindricaeque, aliquando elongatae (5 — 9) × (7 — 13 — 28) μ, singulae, binae, in ramosis aut in catenatae. Post dies 3, pellicula, sicca, non nitida, crispulata, sursum repens formatur. Sedimentum.

Cultura in agar malti (post unum mensem 17°C.) griseola-subfusca, parum acclivis, plana aut parum crispulata, non nitida; margine lobiformo, eroso aut piloso.

Pseudomycelium primitivum abundat.

Fermentatio nulla.

Glucosum, galactosum (lente), L-sorbosum (lente), ethanolum, glycerolum, DL-acidum lacticum et acidum succinicum assimilantur at non maltosum, saccharosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum, melizitosum, D-xylosum, D-arabinosum, L-arabinosum, D-ribosum, L-rhamnosum, *i*-erythritolum, adonitolum, dulcitolum, D-mannitolum, D-

sorbitolum, α-methyl-D-glucosidum, salicinum, kalium D-gluconicum, acidum citricum nec *i*-inositolum.

Ethylaminum hydrochloricum assimilatur at non kalium nitricum nec natrium nitrosum.

Arbutinum aliquando finditur.

Ad crescentiam vitaminae externae non necessariae sunt.

Crescentia potest in 37°C.

Isolata ex cellis ubi vinum praeparatur, *Sorghum* aqua perfusum donec germinaverit et ex aedificiis ad cerevisiam Afram coquendam exstructis.

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LE CONTROLE D'ACTIVITÉ DU VACCIN ANTIPOLIOMYÉLITIQUE[1]

by

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(Reçu le 1 mars, 1961).

Dans cet exposé de l'état actuel des controles du vaccin antipoliomyélitique nous nous limiterons au controle du pouvoir antigénique du vaccin. L'expérience de plusieurs années a, en effet, démontré que les normes de controle d'innocuité, adoptées par la majorité des pays apportent toute garantie de sécurité.

En ce qui concerne la détermination de la valeur antigénique, il est regrettable que les tentatives de standardisation des méthodes de controles n'aient pas encore abouti. Cette situation explique les différences importantes relevées lors de l'examen de préparations de vaccin, considérées comme acceptables sur la base de méthodes imprécises (Org. mond. Santé Sér. Rapp. techn. 145, annex 2–1958; De Somer 1959a).

L'utilisation de ces méthodes, inadéquates pour le controle, est responsable des échecs de certaines campagnes de vaccination, au cours desquelles des préparations de vaccin d'antigénicité insuffisante ont été largement utilisées (De Somer, 1959a).

En corrélation avec le problème du controle d'activité, il faut également considérer le problème de la stabilité des préparations de vaccin. L'absence de test reproductible et significatif n'a pas permis de déterminer sur des bases objectives la durée de validité du vaccin et la stabilité de celui-ci dans différentes conditions de conservation, telle que la combinaison à d'autres antigènes.

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[1] Communication présentée au 6e Congrès de Standardisation Microbiologique, Wiesbaden, Septembre 1960.

Nous avons montré précédemment (DE SOMER *et al.*, 1960) que la vaccination du singe et la comparaison des moyennes des taux d'anticorps à un sérum de référence ne représentait pas une méthode applicable à l'évaluation du pouvoir antigénique du vaccin. La variabilité individuelle de cet animal, le nombre limité d'animaux utilisables et la non-reproductibilité des essais successifs enlèvent toute valeur à cette méthode comme moyen de controle de l'activité du vaccin.

L'introduction de la méthode sur cobayes (GARD *et al.*, 1956) a réalisé un progrès important dans ce domaine. Toutefois la détermination du coefficient d'extinction sur la base d'un dosage qualitatif des anticorps nécessite environ 50 % d'animaux en plus pour obtenir une précision comparable à celle d'une méthode basée sur un dosage quantitatif [1]). De plus l'écart-type de la méthode sur cobayes est de 0.27 $\log_{10}$ (LYCKE, 1958), c.à.d. que l'intervalle de confiance entre les valeurs extrêmes possibles du coefficient d'extinction obtenu est de 1.08 $\log_{10}$. Nous avons mis au point une technique quantitative de dosage permettant d'exprimer la valeur antigénique d'un vaccin par rapport à une préparation standard. Les conditions précises dans lesquelles le poulet pouvait être utilisé dans ce test comparatif ont été déterminées. La méthode basée sur l'existence d'une relation linéaire entre la masse antigénique injectée et la réponse sérologique du poulet, représente une application de l'analyse des régressions des relations log-dose et effet (EMMENS, 1948; LISON, 1958). Les principes généraux de la méthode ont fait l'objet de publications antérieures (DE SOMER, 1959b; DE SOMER *et al.*, 1958, 1959; PRINZIE *et al.*, 1959).

Au terme d'une expérience pratique, portant sur plus de 3 ans il nous a paru utile de présenter les résultats acquis au moyen de cette méthode et de montrer comment il est possible d'en extraire une méthode simplifiée, susceptible de devenir un test de controle de routine. Cette méthode a également été utilisée pour évaluer la durée de validité du vaccin antipoliomyélitique dans diverses conditions.

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[1]) Relation d'efficience entre "median sample" (P.50) avec N_1 animaux et "mean sample" avec N_2 animaux; $0.637\ N_1 = N_2$.

La précision obtenue avec 30 animaux pour N_2, nécessite l'utilisation de 48 animaux pour N_1.

A. ETUDE STATISTIQUE DE LA RÉPRODUCTIBILITÉ DU TEST.

1. Description du test et appréciation statistique.

Le principe de base de la méthode est l'existence d'une relation linéaire entre le log des dilutions de vaccin injecté et le log des réponses sérologiques correspondantes. Les données théoriques ont montré qu'il était possible de comparer la valeur antigénique de deux préparations de vaccin à partir des relations log dose-effet. Ces fonctions linéaires possèdent une inclinaison parallèle, caractéristique de l'antigène poliomyélitique, et la distance horizontale séparant les deux droites exprime la valeur du rapport d'antigénicité entre les deux préparations. Ce dosage biologique doit répondre aux conditions suivantes:

— nombre égal et suffisant d'animaux par dilution de vaccin.
— nombre égal de dilutions de vaccin pour les deux préparations.
— différences égales entre les log des dilutions de vaccin.

L'essai peut être réalisé en 4 points ou en 6 points en utilisant deux ou trois concentrations de vaccin pour chacune des préparations à tester. Les résultats présentés dans cette étude portent sur des tests réalisés en 6 points. En effet, nous avons montré que cette dernière méthode offrait des avantages de précision statistique (PRINZIE *et al.*, 1959).

L'application pratique de la méthode a été facilitée par l'utilisation du poulet comme animal d'expérience. Cet animal, disponible en groupes homogènes importants, se caractérise par l'apparition rapide d'anticorps à titre élevé après une seule injection d'antigène poliomyélitique. Pratiquement, des groupes de 30 poulets de 3 semaines (Rhodes Island Reds ou Hollands Blauw) reçoivent une dose unique de vaccin à la concentration de 1/4, de 1/32 ou de 1/256, pour chacune des préparations. Les sérums individuels de ces 6 groupes d'animaux prélevés au 6e jour, sont séroneutralisés à la dilution unique de 1/8 envers des concentrations décroissantes de virus, de 10^6 à 10^1 IDTC 50. Cette méthode de séroneutralisation représente un gain de temps appréciable, elle exprime la réponse en anticorps par la quantité de virus neutralisé.

Pour chacun des groupes d'animaux la somme des taux d'anticorps (exprimés en $\log_{10}$ de virus neutralisé) est obtenue en additionant les produits du nombre d'animaux possédant des anticorps par la concentration de virus correspondante. A partir de ces résultats une formule générale permet de calculer le rapport d'activité antigénique entre les deux préparations de vaccin:

$$M = \frac{KID}{B}$$

dans laquelle

M = valeur (log) du rapport d'antigénicité entre les deux vaccins.

K = coefficient factoriel, fonction du nombre de concentration de vaccin utilisées dans le test. Dans le cas de l'essai en 6 points ce coefficient est de $\sqrt{8/3}$.

I = intervalle (log) entre les concentrations de vaccin utilisées. Dans le cas présent, cet intervalle est de 0.9 $\log_{10}$.

D = paramètre associé à la différence entre les réponses sérologiques moyennes aux deux préparations.

B = paramètre associé à la différence entre les réponses sérologiques des deux concentrations extrêmes de chacun des vaccins. Cette valeur est proportionelle à l'inclinaison (b) de la fonction linéaire.

Les valeurs D et B sont obtenues par une analyse factorielle des 6 sommes de produits exprimant les effets des concentrations du vaccin administré. Les méthodes de calcul de ces valeurs et la formule générale ont été exposées en détail dans un rapport présenté à la 5e Rencontre Internationale de Standardisation Biologique (Prinzie *et al.*, 1959).

Nous avons constaté que pour des vaccins ne différant pas de façon excessive, l'erreur type de M est environ de 0.14 $\log_{10}$. Dans ces conditions le rapport entre les deux valeurs extrêmes possibles de M est environ de 0.5 $\log_{10}$ pour une probabilité de 0.05, c.à.d. que dans 95 % des cas lors de la répétition d'un même test, les résultats de comparaison ne peuvent différer de plus de trois fois.

De façon empirique, nous avons controlé la reproductibilité du test en comparant à divers moments deux vaccins connus. Le tableau 1 montre, qu'au cours de tests répétés, la différence entre 2 vaccins reste dans les limites de précision de la méthode.

2. Intervalle de confiance et limites de validité du test.

Afin de déterminer les limites entre lesquelles la valeur M du rapport d'antigénicité conserve une valeur significative, nous avons recherché l'intervalle de confiance de M dans 110 tests d'antigénicité. Dans ce but nous avons recherché les écarts-type de M (S_M) dans chacun de ces tests. Une première constatation est que S_M représente une valeur assez constante, liée à la valeur absolue de M.

TABLEAU 1.

Résultats des évaluations du rapport d'antigénicité entre deux vaccins au cours de tests répétés à divers moments.

Vaccins	Date	Rapport d'antigénicité (antilog M) [1]		
		type I	type II	type III
A et B	29—12—59	1.17	0.64	1.06
	26— 4—60	1.34	0.93	1.24
	22— 3—60	1.07	0.60	0.97
	3— 2—60	0.80	0.50	0.50
	3— 5—60	0.80	0.60	0.72
	19— 1—60	0.80	1.26	1.14
C et D	10— 3—60	0.72	1.75	0.35
	5— 4—60	0.72	0.83	0.65
E et F	4— 5—60	0.085	0.66	0.50
	8— 8—60	0.186	1.92	0.94

[1] $M = \frac{KID}{B}$, antilog M = rapport d'antigénicité.

Pour des vaccins différant de 0 à 2 fois, la moyenne de S_M est de 0.14 $\log_{10}$ (avec des quartiles de 0.11 à 0.16 et un écart interquartile 50 % de 0.05) et l'intervalle de confiance de M est de 0.56 $\log_{10}$. Pour des vaccins variant de 2 à 20 fois le S_M moyen est de 0.18 $\log_{10}$ (avec des quartiles de 0.13 et 0.22 ou avec un écart interquartile 50 % de 0.09) et l'intervalle de confiance de M est de 0.72 $\log_{10}$. Lorsque l'écart entre les vaccins augmente, le S_M et par conséquent l'intervalle de confiance, augmente parallèlement. Pour des vaccins variant de 20 à 100 fois le S_M moyen atteint 0.44 $\log_{10}$ c.à.d. que le rapport entre les valeurs extrêmes possibles de M est environ de 50. Il est évident qu'à ce moment les intervalles de confiance élevés ne permettent plus de conclure de façon valable. En conclusion, il apparaît que pour conserver la validité du test il est indispensable de comparer des préparations de vaccins ne différant pas de façon excessive. En pratique, le test ne sera considéré comme significatif que si la valeur antilog de M se situe entre 1 et 0.05, c.à.d. si le rapport d'activité entre les vaccins ne dépasse pas 20. A ce moment l'intervalle de confiance maximum entre les valeurs observées peut varier de 3 à 5 (Tableau 2).

De façon théorique nous avons examiné les divers facteurs intervenant dans les variations du S_M, de façon à déterminer l'importance de chacun de ces facteurs dans la valeur de l'intervalle de confiance

TABLEAU 2.

Intervalles de confiance du test d'antigénicité en fonction du rapport d'activité antigénique entre les préparations de vaccin.

	Rapport d'activité antigénique entre les vaccins (val. antilog de M)	Valeur moyenne de l'écart-type (S_M)	Interv. moyen de confiance du rapp.	
			$\log_{10}$	v-antilog
Tests significatifs	de 0 à 2	0.14 (0.11—0.16) 1)	0.56	3.63
	de 2 à 20	0.18 (0.13—0.22)	0.72	5.25
Tests non-significatifs	de 20 à 100	0.44	1.76	57.5

1) quartiles 50 %.

de M. La formule permettant de déterminer l'écart-type de M est la suivante:

$$S_M = \frac{SKI\sqrt{D^2 + B^2}}{B^2}.$$

Les principaux paramètres ont été décrits précédemment. Le facteur S représente l'écart-type de l'erreur, il exprime le degré d'homogénéité de la réponse des animaux à chacun des vaccins. La détermination de cette valeur dans les 110 tests montre que l'écart-type de l'erreur est relativement constant, sa valeur moyenne est de 1.52 avec des quartiles de 1.35 et 1.69 et un écart interquartile de 0.34. L'homogénéité des groupes est donc suffisante et ce facteur n'intervient pas de façon importante dans la variation du S_M.

Nous avons vu que le paramètre B^2 était proportionnel à l'inclinaison (b) de la relation log dose-effet. La valeur moyenne de cette inclinaison est de 1.264 avec des valeurs extrêmes (95 %) de 1.684 à 0.844. Les valeurs extrêmes correspondantes de B^2 sont environ de 30 à 300. Ces valeurs extrêmes se rencontrent pour des préparations de vaccins se trouvant à la limite de la zone de linéarité de la fonction linéaire liant la masse d'antigène à la réponse sérologique. Ces limites ont été décrites dans une publication précédente (Prinzie *et al.*, 1959). La constance de cette inclinaison est confirmée par une expression de la variance dans laquelle la variation de b est obtenue au moyen du carré moyen de l'erreur.

$$V_b = \frac{S^2}{SSnp \times p^2}.$$

Cette équation montre le même intervalle de confiance de la valeur moyenne de l'inclinaison.

Le paramètre D^2 représente la variable la plus importante dans la détermination du S_M, il est directement proportionnel à la différence entre l'activité des préparations testées. Pour des valeurs antilog de M variant de 1 à 0.05 (vaccins variant de 0 à 20 fois) les valeurs de D^2 se trouvent environ entre 0.1 et 100. Dans ce cas le S_M n'excède pas 0.18 et le test est valable. Le fait de trouver une valeur de D^2 en dehors de ces limites indique que la valeur de M et par conséquent de S_M se trouve en dehors des limites acceptables pour un test valide.

3. Établissement du nomogramme.

Après avoir étudié les paramètres de la formule du rapport d'antigénicité (M) et de son écart-type (S_M) il apparaît que, en conditions normales, certaines de ces valeurs se trouvent dans des limites déterminées et que d'autres restent constantes au cours de tests répétés. A partir de ces données nous avons établi un test simplifié en 3 points, réalisé au moyen de 3 groupes de 30 poulets dont 2 reçoivent des concentrations différentes de vaccins inconnus, et un groupe reçoit une concentration unique du standard. La détermination du rapport d'antigénicité se fait rapidement à partir des valeurs D^2 et B^2 au moyen d'une méthode graphique. Le nomogramme ainsi établi est représenté dans la fig. 1. Ce même graphique permet de vérifier si la valeur M ainsi calculée présente un intervalle de confiance acceptable. Un tel test, utilisant 90 animaux et possédant des caractéristiques de précision bien déterminées pourrait être utilisé comme test de controle de routine pour le vaccin antipoliomyélitique.

B. STABILITÉ DU VACCIN.

La mise au point d'un test d'antigénicité reproductible et statistiquement significatif, dans des limites déterminées, permet d'entrevoir deux applications pratiques de cette méthode. En premier lieu il est important de définir les limites de validité du vaccin en fonction de sa stabilité dans le temps. En second lieu il est utile de vérifier si la méthode est applicable au cas particulier du controle de vaccins combinés contenant l'antigène poliomyélitique.

En ce qui concerne la stabilité du vaccin antipoliomyélitique, nous avons étudié la cinétique de la dégradation antigénique à diverses températures. Des échantillons de vaccin ont été conservés

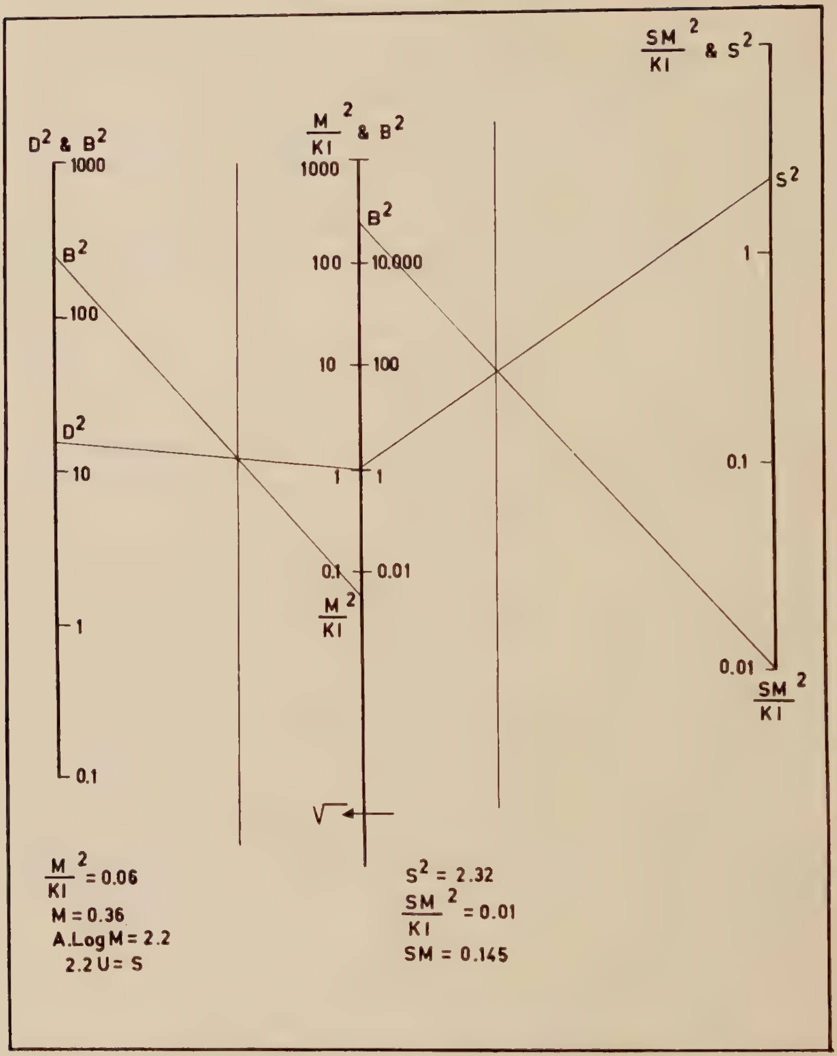

Fig. 1.

à 4°, 24° et 37° durant des périodes variables et l'activité antigénique a été comparée par rapport à un vaccin de référence.

En fonction du degré de perte antigénique nous avons recherché la constante d'inactivation thermique au moyen de la méthode de dégradation accélérée de Jerne et Perry (1956). Selon ces auteurs la constante de dégradation (K) pour une température donnée (T) est en relation avec la valeur antigénique résiduelle (a—x) après un temps d'exposition en secondes (t) à partir d'une valeur antigénique initiale (a).

$$K_T = \frac{1}{t} \log \left(\frac{a}{a - x} \right).$$

Le tableau 3 montre les valeurs K_T obtenues pour le vaccin antipoliomyélitique à diverses températures.

TABLEAU 3.

Résultats des tests de "dégradation accélérée" du vaccin antipoliomyélitique exprimant la constante de dégradation thermique [1]) (K) pour diverses temperatures.

Température (°C)	Temps de conservation	Activité résiduelle (%)	Log K	Moyenne log K
4 °	12 mois	50 %	—8.0197	
	30 mois	10 %	—7.8954	—7.9575
24 °	10 jours	86 %	—7.1203	
	20 jours	73 %	—7.1045	
	30 jours	55 %	—6.9983	
	72 jours	30 %	—7.0753	—7.0746
37 °	10 jours	70 %	—6.7462	
	15 jours	50 %	—6.6339	
	20 jours	25 %	—6.4584	
	35 jours	12 %	—6.5162	
	45 jours	8 %	—6.5514	—6.5812

[1]) $K = \frac{1}{t} \times \log \left(\frac{a}{a - x} \right)$ selon Jerne et Perry (1956).

Lorsque les valeurs log K sont rapportées dans un système de coordonnées, ayant en absisses les valeurs réciproques des températures absolues utilisées, il apparaît que les points trouvés pour les T° de 4°, 24° et 37° se trouvent approximativement sur une ligne droite. Une relation comparable a été décrite pour l'anatoxine tétanique par Cohen *et al.* (1959).

Par extrapolation, il est possible d'estimer le temps nécessaire pour que le vaccin perde 50 % de son activité à une température donnée de conservation. Il est évident à partir de ces résultats que la durée de validité du vaccin ne peut dépasser un an, puisque à ce moment le vaccin conservé à 4° ne représente plus que la moitié de l'activité antigénique initiale (fig. 2).

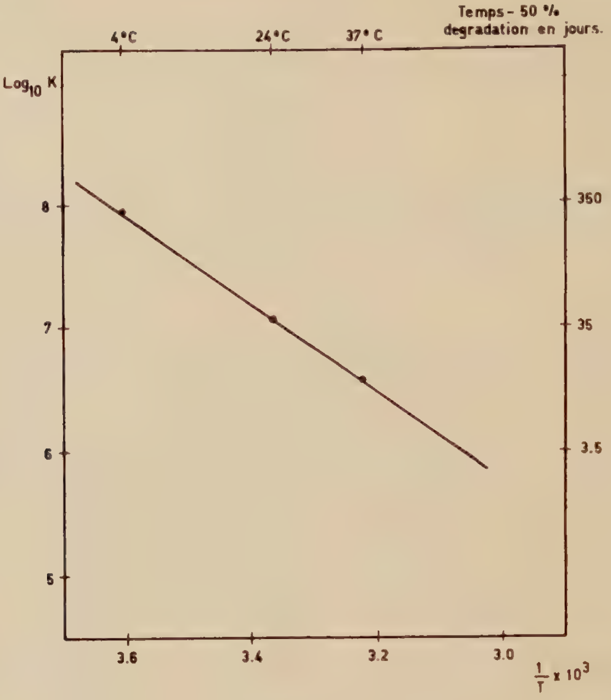

Fig. 2.

Relation entre les valeurs de log K et les valeurs réciproques des températures absolues correspondantes. Estimation du temps de conservation entraînant 50% de dégradation du vaccin.

Avant d'étudier la stabilité de l'antigène poliomyélitique en combinaison avec d'autres antigènes, nous avons recherché si la méthode décrite était applicable à l'antigène combiné. Dans ce but nous avons examiné si la présence d'aluminium pouvait modifier la réponse sérologique du poulet. Tant sur singes que sur poulets, l'absorption sur aluminium ne modifie pas de façon significative la réponse sérologique. Ce point étant établi, nous avons ensuite comparé des lots commerciaux de vaccin combiné, prélevés durant leur période de validité, avec un vaccin combiné fraîchement préparé et un vaccin antipoliomyélitique trivalent, de l'anatoxine diphtérique et tétanique et une suspension de *Bordetella pertussis*.

Le tableau 4 montre que ces préparations possèdent des activités antigéniques très différentes en ce qui concerne l'antigène poliomyélitique. Ces différences ne sont pas dues à la présence de l'aluminium absorbant mais bien à l'état de pureté des autres antigènes présents dans la combinaison. En effet, les résultats obtenus en mélangeant le poliovaccin avec diverses préparations d'antigène diphtérique et tétanique ont montré qu'en présence de préparations impures, l'antigène poliomyélitique ne présentait pas les caractéristiques normales de conservation.

TABLEAU 4.

Détermination de la valeur antigénique de diverses préparations de vaccins combinés[1]) par rapport au vaccin de référence (R_5).

Vaccins	Rapport d'activité antigénique (antilog M)		
	type I	type II	type III
A	1.34	0.93	1.24
B	0.25	1.78	2.40
C	0.035	0.235	0.33
D	0.004	0.045	0.002
E	0.04	0.14	0.14
F	0.1	0.12	0.001

[1]) Ces préparations de vaccins combinés représentent des lots commerciaux testés au cours de leur période de validité (conservation à 0°C.).

Il résulte de ces données que le vaccin combiné pose un problème de standardisation. Un essai de stabilité, basé sur les méthodes exposées ci-dessus, devrait être effectué pour chacun des types de ce vaccin afin de déterminer la période de validité de ces préparations en ce qui concerne l'antigène poliomyélitique.

Conclusions.

Les limites de validité de la méthode de détermination de la valeur antigénique du poliovaccin ont été vérifiées en se basant sur une expérience de 3 ans. Cette étude a permis de déterminer les limites entre lesquelles un test peut être reconnu comme valable et significatif. En fonction de ces données, un test simplifié est proposé. Dans ce test, le rapport d'antigénicité est établi rapidement au moyen d'un nomogramme.

La méthode utilisée dans notre laboratoire a servi à rechercher le degré de stabilité de l'antigène poliomyélitique. De ces résultats, il ressort que la période de conservation du poliovaccin ne devrait pas dépasser 1 an. Au terme de cette période, le vaccin a perdu environ 50 % de son activité antigénique initiale. La combinaison de l'antigène poliomyélitique à d'autres antigènes et son absorption sur aluminium n'interfèrent pas avec la technique de détermination. La stabilité des vaccins combinés est variable, il est indiqué d'effectuer des essais de stabilité de ces préparations afin d'obtenir une standardisation de l'activité de l'antigène poliomyélitique dans ces vaccins.

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A TISSUE CULTURE COLOR TEST FOR THE TITRATION OF NEUTRALIZING ANTIBODIES AGAINST SENDAI VIRUS (PARAINFLUENZA 1)

by

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Phenol red is added as a pH indicator to the tissue culture media used in virological laboratories. During the metabolism of the cells acid byproducts are formed and released into the medium. Generally within a few days the phenol red indicator will turn yellow.

In 1949 ENDERS observed that poliovirus had a marked influence on the metabolism of cultures of human embryonic cells, infected cultures producing less acid than the non-infected controls. However, the variable conditions in the tissue cultures resulted in inconstant pH changes, so that the change in color of the medium could not be used as a dependable criterium. Better results were obtained by observing cell destruction.

SALK, YOUNGNER and WARD (1954) devised a method, by suitable adjustment of tissue concentration and of buffer, that allowed the use of the color change of the phenol red as an indicator of virus activity. The color test they developed is based on the principle that a certain cell concentration in a certain amount of buffer will turn the color of the medium from red to yellow in a certain time. When poliovirus is added to the cell suspension, prior to incubation, the virus will destroy the cells just before the metabolism is sufficiently established to change the pH of the medium.

The special merit of this Salk color test lies in the fact that it offers a relatively simple method of titrating neutralizing antibodies against poliovirus. A dilution series is made of each serum and mixed with a dose of poliovirus. After incubation the mixture is added to the cell suspension. Positive neutralization is indicated by metabolism of the cells — the medium turning yellow, while negative tubes remain red.

GAUSH and YOUNGNER (1959) devised a similar test for the titration of influenza antibodies.

Essential for the success of a color test with other viruses is the question whether the virus used is acting quickly enough on the metabolism of the cell suspension.

During our experiments with the Sendai virus (parainfluenza 1) we observed that the characteristics of this virus in tissue culture — regarding the microscopic aspect of the cytopathogenic effect and the quick, almost explosive, destruction of the monolayer — closely resembled those of poliovirus. Thus an attempt was made to use the Sendai virus in the color test.

MATERIALS AND METHODS.

The virus.

The Sendai virus strain used was received in a freeze dried state through the courtesy of Dr. C. H. ANDREWES (London).

The virus was propagated by serial passage in the allantoic sac of 11 day old chick embryos (white leghorn). The eggs were inoculated with virus diluted 1 : 1000, incubated for three days, chilled at 4°C. during 5 hours and then the allantoic fluid was harvested.

The virus was pooled and stored at —20°C. in small glass tubes.

The cell suspension.

Minced kidney tissue from cynomolgus monkeys was trypsinized with 0.25 % trypsin (Difco) in phosphate buffer solution. The cells were washed once with Hanks balanced salt solution and the sediment resuspended in a medium consisting of Hanks balanced salt solution containing 0.5 % lactalbumin hydrolysate and 5 % horse serum, to which 100 U of penicillin, 100 U of streptomycin, and 50 U of mycostatin per ml were added.

A sample of the heavy cell suspension was drawn and after staining with a solution of brilliant cresyl blue (Groma Gesellschaft) a cell count was performed in a Buerker-Tuerk chamber. For use in the color test the cell suspension was diluted with medium to 250.000 per ml.

The color test.

The color test was performed in glass tubes measuring 55 × 8 millimeter. A twofold dilution series of serum was made in medium in volumes of 0.25 ml. To each tube 0.25 ml of virus dilution in medium, containing approximately 100 color test doses, were added. The mixture was incubated for 1½ hour at room temperature (20°C.) to allow neutralization. Hereafter 0.25 ml of the cell suspension was added and the fluid in the tubes was covered by 0.25 ml heavy sterile paraffin oil.

In each color test the following controls were made:

a) with each serum control tubes without virus were used, in order to exclude possible toxic effect of the serum on the cells, which can mask neutralization;

b) control tubes containing a cell titration and

c) a titration of the virus.

The color test was read after incubation at 37°C. for one week.

The sera.

Antisera were prepared by immunizing young healthy rabbits, which received 3 intraperitoneal injections of 10 ml of virus with an interval of 2 weeks. Chicks were immunized by three subcutaneous injections of 5 ml of virus with an interval of 2 weeks. Ferrets were immunized by one intranasal instillation of virus.

The receptor destroying enzyme.

Most of the sera used for the haemagglutination inhibition test against Sendai virus show a slight non-specific inhibition which can be removed by treatment with cholera filtrate R.D.E. Preliminary tests had shown that non-specific inhibitors may yield false positive results indeed.

The sera were treated by adding 5 parts of cholera filtrate to 1 part of serum, incubating overnight and inactivating the mixture at 56°C. for one hour.

The cholera filtrate used was produced in our biochemical department.

It proved to be important to use a colorless brand of peptone in order to prevent discoloration of the medium in the tubes. Some batches of cholera filtrate showed some toxic effects on the monkey kidney cells. To avoid misresults the cholera filtrate to be used was tested in preliminary color test.

Commercial cholera filtrate proved to be toxic for the cells, as a result of addition of preservatives.

RESULTS AND DISCUSSION.

To ascertain the specificity of the reaction, the Sendai color test was performed with sera from animals immunized against Sendai virus, mumps virus, Newcastle disease virus and parainfluenza 2 virus, the results of which are given in table 1.

TABLE 1.

Titers of animal immune sera in the color test with Sendai virus.

Animal	No.	Immunized against	Color test titer
Rabbit	114	Sendai	512
Rabbit	195	Sendai	512
Rabbit	147	Mumps	<6
Rabbit	148	Mumps	<6
Rabbit	68	Newcastle	<6
Ferret	25	Paraflu 2	<6
Chicken	59	Sendai	512
Chicken	60	Sendai	256
Chicken	61	Sendai	320
Chicken	62	Sendai	320
Chicken	53	Mumps	<6
Chicken	54	Mumps	<6
Chicken	58	Newcastle	<6

The Sendai color test proved to be highly specific within the group examined, no cross reactions with antibodies against the other viruses of this group were observed.

In both the haemagglutination inhibition test and the complement fixation test DE MEIO and WALKER (1957) observed antibody rises against Sendai virus in mumps patients, but they stated that no cross reactions between Sendai and mumps were detectable in rabbit or mouse sera following injection of mumps virus. In our experience however, the rabbit mumps immune sera showed a Sendai titer and the Sendai immune sera a mumps titer, although in both cases these titers were markedly lower.

For the serological diagnosis of infections with Sendai and mumps virus it would be important to give a more clear answer than is possible by the haemagglutination inhibition test (h.i.t.) and the complement fixation test (c.f.t.) which show marked cross reactions. In order to answer the question whether the color test with Sendai virus is sufficiently specific, a group of 75 human sera chosen at random was taken. With these sera the h.i.t. and the c.f.t. for mumps and Sendai virus and the color test with Sendai virus were performed. The results are given in table 2.

TABLE 2.

Reaction to mumps and Sendai viruses in the haemagglutination inhibition test, the complement fixation test and the color test of 75 human sera taken at random.

Reaction type	h.i.t. mumps	h.i.t. Sendai	c.f.t. mumps	c.f.t. Sendai	no. of sera in the group	no. of sera positive in color test
1	+	+	+	+	5	4
2	+	+	+	—	2	1
3	+	+	—	—	7	3
4	+	+	—	+	2	1
5	+	—	+	+	1	1
6	+	—	+	—	7	0
7	+	—	—	—	9	0
8	—	+	+	+	1	1
9	—	—	+	+	0	0
10	—	—	—	+	0	0
11	—	+	—	+	5	5
12	—	+	—	—	8	3
13	—	+	+	—	2	2
14	—	—	+	—	5	0
15	—	—	—	+	0	0
16	—	—	—	—	21	2

The most striking feature in table 2 is the fact that reaction types 6, 7 and 14, which represent the typical mumps group, are completely negative in the Sendai color test.

In reaction type 11, representing a typical Sendai group, all sera are positive. Within the sera of reaction type 12 (h.i.t. with Sendai positive), only 3 showed a positive color test, 5 being negative. It may be possible that the latter are due to remote infections and that the color test is not sensitive enough to detect such low antibody levels. The reverse may be the case with the 2 sera positive in group 16.

In the other groups (1, 2, 3, 4, 5, 8, and 13) the h.i.t. and the c.f.t. are inconclusive for distinguishing between mumps and Sendai infection. In these cases, however, the results of the color test might be conclusive to differentiate between the two infections, assuming that all color test positive sera must be ascribed to previous Sendai infection.

Summary.

A color test for the detection and titration of neutralizing antibodies against Sendai virus is described. Non-specific inhibitors should be removed by treatment with cholera filtrate R.D.E. In contrast to both the haemagglutination inhibition test and the complement fixation test, which do not allow a differentiation between mumps and Sendai virus infection because of cross reactions, the color test is assumed to be sufficiently specific for Sendai virus infection.

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SEROLOGICAL TYPING OF MENINGOCOCCI BY MEANS OF MICRO-PRECIPITATION

by

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Since the early years of this century, gels have been used for the diffusion and precipitation of antigens and antibodies. PETRIE (1932) was the first to employ this technique for the typing of meningococci.

He demonstrated that, around colonies of meningococci grown on a nutrient medium which contains immune serum, precipitates appear in the form of rings which are known as halo's. A halo represents a specific precipitate, formed as a result of the action of serum antibodies on the specific polysaccharide of the meningococci. The polysaccharide of the microorganisms is liberated from the bacteria and diffuses into the agar surrounding the colony. The diameters of the rings surrounding colonies of group I meningococci remain relatively constant in size during the first three days, but subsequently show enlargement. The explanation is that the specific precipitate is dissolved when one of the reagents is present in an excessive quantity, which is due to growth and autolysis of the colony.

It was found that meningococcal colonies form no halo when grown on agar containing normal or anti-pneumococcal serum.

With the aid of this method, PETRIE was able to classify strains of meningococci in accordance with the division then used (groups I, II, III and IV), but encountered considerable difficulties due to partial antigenic identity.

Furthermore he observed that old meningococcal strains produced less type-specific polysaccharides than strains of recent isolation; the older strains formed less marked rings or none at all. RAKE (1933) was also unable to obtain specifically precipitating sera with old strains. He assumed, like PETRIE, that frequent subcultures of meningococcal strains caused diminution or loss of substances capable to produce specific precipitation.

In contrast, KIRKBRIDE and COHEN (1934) demonstrated that precipitating sera can be obtained with old strains of groups I and III, although these strains were isolated more than fifteen years previously. They were unable to demonstrate a difference in precipitation between old and recently isolated strains of groups I and III. However, strains of groups II and IV generally lacked the substance provoking specific precipitins; with these strains they obtained only sera which yielded very poor or no precipitation at all. This was true for both old and freshly isolated strains.

PITTMAN, BRANHAM and SOCKRIDER (1938) demonstrated a strict correlation of type-specific precipitins in a serum and of the protective influence of this serum in mice. These authors used the method of PETRIE. They found that only a few of the immune sera against group II meningococci yielded a slight halo around the colonies of this type.

After the development of OUCHTERLONY's double diffusion method in two dimensions [1]), JYSSUM (1958) used this method in an investigation comparing indirect hemagglutination and gel precipitation of three meningococcal types. He demonstrated *inter alia* that three strains representing group B, group C and a subtype of group B, contain various soluble fractions which he identified as different antigens by means of gel precipitation.

OWN PRECIPITATION TESTS WITH MENINGOCOCCI.

With the aid of the macro-technique of the double diffusion method in two dimensions, we have tried to obtain specific meningococcal precipitation lines. We have partly succeeded: specific precipitation lines were obtained with sera of groups A and C (formerly the groups I and II α) and strains of these groups.

The great disadvantage of this macro-technique in Petri dishes is the long time interval between the filling of the perforations with antigen and antiserum and the appearance of the precipitation lines. The lines are not clearly visible until after several weeks, and this represents a serious objection to practical application.

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[1]) For a description of the history of precipitation tests in general, we refer to the survey presented by OUCHTERLONY (1958).

Since 1959 we have used a micro-technique based in principle on that used in the Central Laboratory of the Blood Transfusion Service, Amsterdam in the immunochemical investigation of serum proteins (investigation of F. PEETOOM).

This micro-technique was described among others by GRASSET, PONGRATZ and BRECHBUHLER (1956) in a study of venoms and anti-venomous sera.

Instead of Petri dishes we now use microscope slides or lantern slides (5 × 5 cm). A layer of melted agar is placed on the slide and, when the agar has become firm, perforations are made in the agar according to the pattern required, by means of suction. The method involves the filling of the central perforation with antiserum with the aid of capillaries; the peripheral perforations are filled similarly with a very thick meningococcal suspension in 0.9% NaCl solution. The result can be read after 18 hours, which represents a considerable improvement compared with the macro-method. Another advantage is that only minimal quantities of antiserum and smaller quantities of meningococcal suspension are required.

We are aware of the fact that use of a suspension of meningococci means that we are dealing with a number of antigens, some of them being specific and others non-specific antigens. We did not prepare extracts from meningococci, which should have provided us with antigens in a purer form.

Also we admit that use of meningococcal suspensions may give rise to difficulties caused by quantitative variables.

However the results with the strains of the various types were very constant and we find our technique useful for epidemiological purposes.

A detailed description of this technique and of the preparation of the antigenic suspensions and the antisera follows at the end of the report.

We found that meningococci of the various types have antigens in common, but apart from this, A and C strains give rise to specific precipitation lines. Absorption of the precipitating A and C sera with suspensions of strains not belonging to A and C strains respectively, did not remove these specific precipitation lines. However, after absorption of these sera with suspensions of the homologous strains, the specific lines completely disappeared.

We did not succeed in obtaining antisera which yielded specific precipitation lines against B strains (formerly group II). We used both standard B strains of S. E. Branham and B strains freshly isolated from patients for preparing the antisera and for performing the precipitation reaction.

With *Neisseria* species other than *Neisseria meningitidis* (*i.e.*, *N. flavescens* and *N. gonorrhoeae*) the various immune sera yielded precipitation lines corresponding to the non-specific meningococcal lines.

It has been observed that more non-specific precipitation lines are formed when the immunization of rabbits was prolonged over a certain period of time, which varied for each individual rabbit.

It has thus become possible to use the precipitation method for typing, besides typing by means of agglutination. This precipitation has the advantage that the result can be lastingly demonstrated.

We have re-typed our stock of A and C strains from patients and carriers with the aid of the microprecipitation technique and obtained in each test results which were identical with the results obtained with the agglutination technique.

An example of an A-strain typing is seen in fig. 1, while an example of a C-strain typing is found in fig. 2.

There remained however a number of strains which were not typable with the agglutinating A, B and C sera and with the precipitating A and C sera [1]).

With three non typable strains isolated from carriers, we succeeded to prepare sera which were specific for each in agglutination and precipitation. We provisionally labelled them type X, Y and Z.

Absorption of the precipitating X, Y and Z sera with suspensions of strains not belonging to these types, did not alter the specific precipitation lines. However after absorption of these sera with suspensions of their own strains, the specific lines completely disappeared.

With the precipitation technique we also tried to type the strains which were not typable because they showed auto-agglutination; these strains only gave rise to non-specific lines with sera of the various types.

Four strains isolated from cerebrospinal fluid belonged to type Y

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[1]) All our strains were also tested for agglutination with a D serum; among over 700 meningococcal strains isolated from patients and carriers, we have never found a strain of type D.

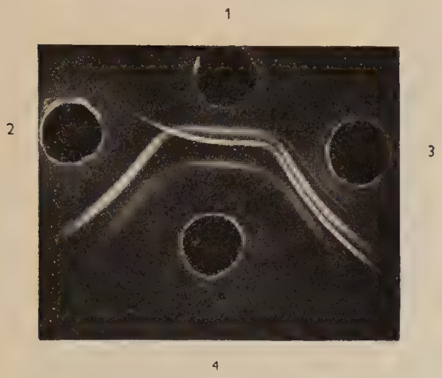

Fig. 1.
1. Patient A strain.
2. Standard C strain.
3. Standard A strain.
4. A antiserum.

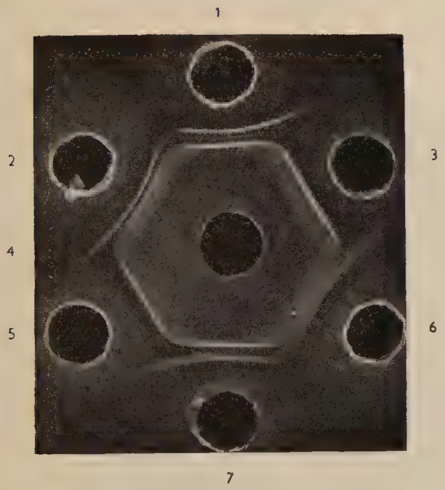

Fig. 2.
1. Patient C strain.
2. Standard C strain.
3. Standard A strain.
4. C antiserum.
5. Standard A strain.
6. Standard C strain.
7. Patient C strain.

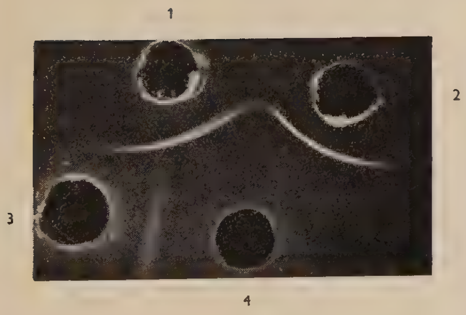

Fig. 3.
1. Patient Y strain.
2. Standard Y strain.
3. Standard Z strain.
4. Y antiserum.

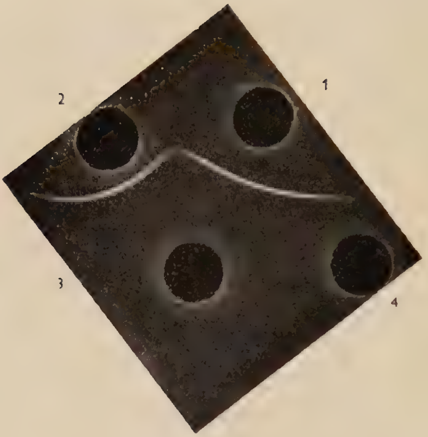

Fig. 4.

1. Carrier X strain.
2. Standard X strain.
3. X antiserum.
4. Standard Y strain.

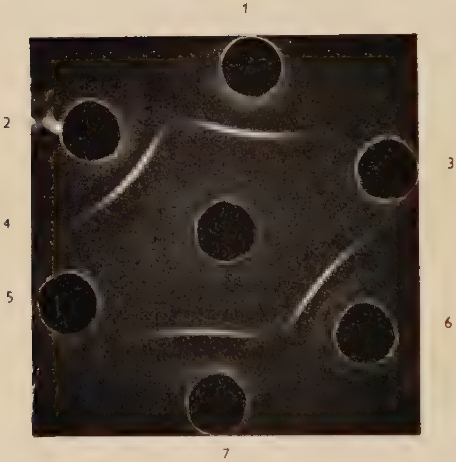

Fig. 5.

1. Carrier Z strain.
2. Standard Z strain.
3. Standard A strain.
4. Z antiserum.
5. Standard A strain.
6. Standard Z strain.
7. Carrier Z strain.

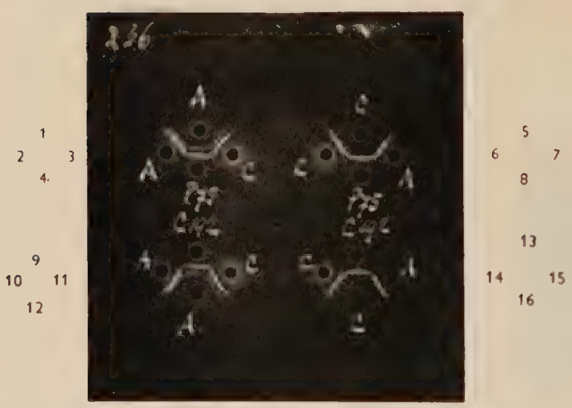

Fig. 6.

1. A antiserum.
2. Standard A strain.
3. Standard C strain.
4. Patient A strain.
5. C antiserum.
6. Standard C strain.
7. Standard A strain.
8. Patient A strain.
9. Carrier A strain. (contact of patient).
10. Standard A strain.
11. Standard C strain.
12. A antiserum.
13. Carrier A strain. (contact of patient).
14. Standard C strain.
15. Standard A strain.
16. C antiserum.

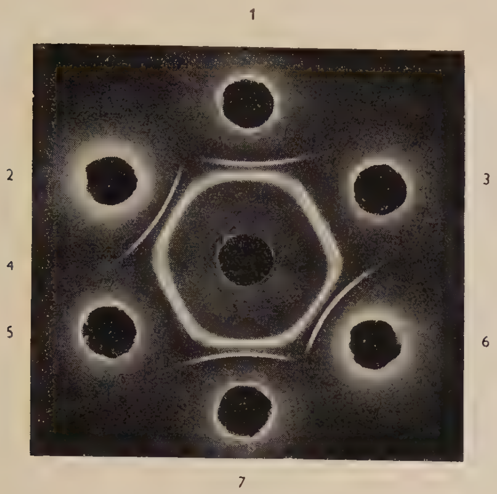

Fig. 7.
1. Patient C strain.
2. Standard C strain.
3. Standard A strain.
4. C antiserum.
5. Standard A strain.
6. Standard C strain.
7. Patient C strain.

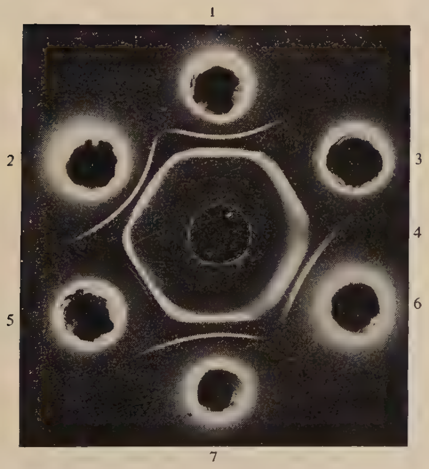

Fig. 8.
1. Carrier C strain.
(contact of patient).
2. Standard C strain.
3. Standard A strain.
4. C antiserum.
5. Standerd A strain.
6. Standard C strain.
7. Carrier C strain.
(contact of patient).

(fig. 3), while another strain obtained from a fifth patient, belonged to type X (fig. 4). The third new type represents a strain isolated only from carriers and has not yet been found among our 120 strains from patients (fig. 5).

These new types X, Y and Z were characterized by the following features such as: Gram negative diplococci, which showed a positive oxydase reaction; clear colourless colonies on ascites-broth agar;

no growth at 22°C. on ascites-broth agar; fermentation of glucose and maltose but not of saccharose.

Recently we received two non-typable meningococcus strains sent to us by Miss E. O. King (C.D.C. Atlanta, U.S.A.). One of these strains turned out to be identical with our type X strain both in agglutination and precipitation reaction. The other strain was not typable with any of our sera. Perhaps there will be more types than we are acquainted with now.

All strains isolated from patients belonged to the types A, B, C, X and Y, while some isolated from carriers belonged to type Z. An example of the typing of A strains from a patient and a carrier (a contact of the patient) is given in fig. 6 (real size).

Fig. 7 and fig. 8 show the typing of C strains from a patient and a carrier (a contact of the patient).

TECHNICAL DATA.

a. Preparation of antisera for precipitation tests.

Agglutinating and precipitating antisera are obtained by injecting a meningococcal suspension in 0.9% NaCl solution (pH 7) into rabbits three times a week. For the preparation of A, B and C antisera we use the standard strains[1]) kindly supplied to us by S.E. Branham. The remaining sera are prepared with three strains isolated by us from carriers. They did not react with the A, B, C and D antisera. All strains are stored dry. We use sheepblood agar (5%) as nutrient medium. We begin by injecting 0.1 ml of a thin suspension (1000×10^6 microorganisms per ml) killed by boiling, after which injections of living suspensions of increasing thickness (up till 3000×10^6 microorganisms per ml) in increasing dosage (up till 1 ml) are applied. The serum agglutinin titre is regularly determined and examinations are made to detect the presence of specific precipitating antibodies. As soon as sufficient agglutinins (titre 1 : > 100) and/or precipitins are available, a cardiac puncture is done. The period of immunization varies but it practically always exceeds a month. The serum is stored at —24°C. and is used undiluted in the precipitation tests.

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[1]) Type A: M 1027, type B: M 993, type C: M 1628, type D: M 158.

b. Preparation of antigens for precipitation tests.

Meningococci grown on sheepblood agar (5%) or ascites broth agar (10%) are used for preparing a very thick suspension in an agglutination tube filled with a few drops of a 0.9% NaCl solution pH 7; the suspension should be so thick as to present the appearance of coffee cream.

c. Detailed description of the microprecipitation technique.

Microscope or lantern slides (5 × 5 cm) are very carefully cleaned with a cloth.

With the aid of a Pasteur pipette, a few drops of a 1% agar (1% Bacto-agar in 0.9% NaCl solution pH 8) are dropped on to a slide and rapidly spread over the entire slide with the finger; the 1% agar is allowed to dry on the slide (best done by putting the slide in the incubator at 37°C. for 1—2 hours).

After drying, it is possible to write on the 1% agar with glass ink.

A 5 ml pipette is then used to apply a layer of 1.5% agar (1.5% Bacto-agar in 0.9% NaCl solution pH 8) to the slide. The slide must meanwhile remain completely horizontal to prevent running. The slide should be covered by an even layer.

The 1% agar layer serves to obtain a better attachment of the 1.5% agar layer, that serves as a diffusion medium on the slide. Furthermore the 1% agar layer prevents, that antigen and antiserum penetrate between the diffusion medium and the slide, by which the precipitation lines would become of a diffuse and irregular form.

The agar is allowed to coagulate by exposure to the air. The slide can be kept for a few days at room temperature in a container closed by a glass plate, and kept humid to prevent desiccation of the agar. A few granules of thymol can be added to counteract mould growth on the agar. Perforations within the agar can be made by means of a dull needle (2 mm diameter) which is connected by means of a plastic tube with a suction apparatus. For this purpose, a model drawn on millimetred paper can be placed underneath the slide. As a rule, an interspace of 5 mm between the perforations is sufficient. It must be ensured that the needle is kept exactly vertical during suction. The holes are filled by capillaries with meningococcal suspensions and antisera. We often use a model of four or six holes arranged around a central hole. The central hole is filled with antiserum, while the peripheral holes are filled with meningococcal suspensions.

The slides are kept in the container for 18—24 hours at room temperature. The lines as a rule become clearly visible after this period.

The slide is then washed with 0.9% NaCl solution for 24—48 hours. After this, the suspensions are removed by spraying with 0.9% NaCl solution.

A filter paper of the size of the slide is then placed on the still moist agar surface of the slide, which is placed horizontally in an incubator and left overnight at 37°C.

After this the filter paper is removed from the dried agar without difficulty and without damaging the dried agar layer.

The slide is then stained with a solution of amido-black [1]), to which it must be exposed for about 15 minutes to obtain well-defined coloured precipitation lines. The stain is washed off thoroughly with distilled water, and the slide is left to dry in the incubator.

If necessary, notes can now be made on the slide with glass ink.

When lantern slides are used, these can be immediately applied in the projection apparatus. A clean slide may be placed on the side of the slide on which the precipitation is shown in order to protect the lantern slide.

Summary.

With the aid of a microprecipitation technique on slides, it is possible to visualise, within 18 hours, precipitation lines specific for type A and C meningococci. The slides can be stored after drying and staining, and can be directly used in projection.

Among a number of strains not typable with the standard sera, three new types were identified by means of agglutination and precipitation. Provisionally they were called type X, Y and Z. Strains of type X and Y were isolated from patients and carriers, those of type Z up till now only from carriers.

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[1]) Amido-black solution. First make buffer: 30 g concentrated acetic acid in 500 ml distilled water and 6.8 g sodium acetate in 500 ml distilled water. To 900 ml of this buffer, add 100 ml glycerine and 0.5 g amido-black.

Acknowledgement.

Thanks are due to F. PEETOOM and Mrs E. BORST-EILERS, for their kindness in demonstrating their microprecipitation method to us. The suggestive remarks by F. PEETOOM are also gratefully acknowledged.

We would like to thank Dr A. COHN for his critical remarks.

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PIGMENTS OF SOME FLAVOBACTERIA, XANTHOMONADS AND CORYNEBACTERIA [1] [2]

by

ROBERT TWAROG [3] and **ROBERT C. CLEVERDON**

(Received March 20, 1961).

Serologic diagnosis of yellow strains of *Flavobacterium*, *Corynebacterium*, and *Xanthomonas* was found of little practical value by Soda and Cleverdon (1959). The relative ease of spectrophotometry of clarified cellular suspensions as described by Barer *et al.* (1953) and Barer (1955), suggested a study *in vivo* of pigments of yellow strains of *Flavobacterium*, *Corynebacterium*, and *Xanthomonas* with an aim of establishing an additional taxonomic criterion.

Materials and Methods.

Cultures. *Flavobacterium acidificum* (F-8366), *F. arborescens* (F-4358), *F. esteroaromaticum* (F-8091), *F. flavoscens* (F-8315), *F. odoratum* (F-4651), *F. suaveolens* (F-958), and *F. vitarumen* (F-10234) were obtained from the American Type Culture Collection; *F. aquatile* (F-700) from the Midwest Service; *F. denitrificans* (F-100) from the Carolina Biological Supply Corporation; *Flavobacterium* sp. (F-48) from Dr. Michael Pelczar, Jr. *Xanthomonas beticola* (X-10304), *X. pelargonii* (X-8721), *X. phaseoli* (X-9563), *X. pruni* (X-10017), and *X. translucens* (X-9000) were obtained from the American Type Culture Collection; *X. pruni* (X-P 13), *X. barbariae* (X-B 1), *X. campestris* (X-C16), *Corynebacterium rathavi* (C-R1) and *C. poinsettiae* (C-P2) were from Dr. Walter Burkholder's collection.

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[1] Supported by Grant E-706, National Institutes of Health, U.S.A.

[2] Contribution number 44 of the Institute of Cellular Biology, Univ. of Conn.

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Cultures were maintained by monthly transfer (and subsequent storage at 4°C.), using slants of the following composition in g/l: casitone, 5.0; NaCl, 5.0; beef extract, 3.0; K_2HPO_4, 2.0; glucose, 1.0; and agar, 18.0; final pH 7.2. This medium without agar was used throughout for growth of cells.

Equipment. Bovine albumin fraction V (lots S14607, S14912, and P13808) was obtained from the Armour Laboratories. A Bausch and Lomb microscope (bright phase) was used for matching of refractive indices of the bacteria and the bovine albumin solutions; a Coleman Junior Spectrophotometer model 6-A was used to establish density of bacterial suspensions for this purpose; a Beckman spectrophotometer model DU (with corex cuvettes) was used for absorbance analyses. This Beckman instrument was standardized with a mercury lamp as outlined by the manufacturer.

Preparation of clarified cell suspensions. Cells were grown at 28°C. from 1 to 15 days in bottles containing about 50 ml of casitone broth shaken reciprocally at 70 cycles per minute, harvested by centrifugation, and washed twice with distilled water. Packed cells were mixed with a fine glass rod in droplets of albumin solutions containing a few granules of emery. Hanging drop examination with the phase microscope (44 × objective) revealed the matching of refractive index of menstruum and cells. Absolute matching (no visible cells) was not always possible, owing to a few clumps. Uniform suspension was often facilitated by a small stirrer (Cleverdon, 1958). Final concentrations of albumin required were from 24.0 to 27.5 per cent (w/w). The clarified suspensions for analysis were then made adding the required albumin to cellular suspensions of $O.D._{520}^{1\,cm}$ 1.2 ± 0.2.

The suspension and albumin blank installed in the cuvette holder, were placed in a pyrex vacuum desiccator. The pressure was reduced (28 inches on a dial gauge) for 10 min., which effectively degassed the suspensions, rendering them suitable for analysis.

Preliminary studies from 950 $m\mu$ to 340 $m\mu$ in 2 $m\mu$ steps indicated that the absorption range was from 600 to 390 $m\mu$.

Results and discussion.

The absorption of the intracellular pigments of all cultures was in general high in the shorter wave lengths, and although some absorptions were intensified in the older cells, no new ones arose.

TABLE 1.

Absorbances (× 10^3) of clarified cellular suspensions of 11 flavobacteria, 9 xanthomonads and 2 corynebacteria. Optical path 1 cm. The cultures were from 4 to 7 days old. The numerical value of the absorbance varies with intensity of pigment of cell and with cellular concentration, so that maxima are shown in bold face.

Wavelength, millimicron	Cultures																					
	F-48	F-100	F-700	F-958	F-4358	F-4651	F-8091	F-8315	F-8366	F-10234	X-B1	X-C16	X-P13	X-7381	X-8721	X-9000	X-9563	X-10017	X-10304	X-R1	C-P2
390	220	615	261	**558**	288	424	**565**	210	384	386	**484**	394	379	512	315	372	615	273	422	371	396
395	217	630	260	560	296	436	556	232	410	424	**482**	406	395	521	343	396	630	284	466	393	406
400	219	632	260	561	**300**	440	550	262	418	435	471	415	**404**	**530**	**355**	**416**	**638**	306	470	**400**	**411**
402	215	636	256	**563**	**300**	449	548	267	420	442	471	**418**	**406**	**530**	**355**	**417**	**638**	310	**473**	**402**	**410**
404	217	636	255	**562**	298	452	540	273	**424**	442	475	**418**	**406**	**528**	**355**	**416**	**638**	311	**472**	**400**	406
406	220	646	256	**560**	293	456	**542**	**270**	**422**	**446**	475	411	395	522	350	407	**638**	**321**	470	396	401
408	220	**650**	260	558	291	462	**545**	**270**	420	**446**	474	404	390	518	345	406	628	306	466	393	395
410	227	**650**	266	552	281	462	540	267	418	**445**	475	395	381	512	339	400	620	302	462	387	391
414	243	645	280	542	272	470	530	252	410	440	470	387	350	502	326	383	610	290	448	374	380
418	247	639	293	530	260	**479**	525	238	401	440	464	378	341	491	313	367	600	270	432	362	370
420	**251**	634	**297**	522	257	**478**	520	230	396	419	459	374	331	486	305	355	592	271	426	353	364
424	**250**	622	**298**	508	246	462	500	215	385	399	499	365	314	460	291	339	580	264	416	341	355
430	240	579	285	478	233	436	488	199	366	358	422	350	287	442	266	310	562	249	402	312	340
450	219	432	254	412	219	315	459	163	294	314	366	274	234	**381**	193	221	470	157	345	252	316
470	171	360	198	363	225	306	431	152	271	275	327	236	203	380	165	193	422	125	330	211	316
476	174	336	220	**368**	233	292	420	152	262	282	312	226	190	336	157	180	410	119	320	**215**	**320**
478	176	326	222	**368**	**235**	284	426	153	258	**285**	306	224	188	335	155	177	410	117	310	**215**	**321**
480	**180**	320	217	363	**236**	279	421	152	254	283	300	221	186	331	153	175	404	117	306	211	**322**
500	125	263	128	288	216	225	400	144	232	223	256	195	160	274	137	156	371	093	285	167	316
550	074	137	107	248	147	148	356	129	154	188	193	153	119	235	111	119	302	076	177	134	256
600	094	094	089	220	076	120	321	123	129	159	161	128	096	206	091	**092**	258	060	144	110	175

The absorption was reproducible, although the absorbance of older cells was greater, and of some cells greater regardless of age, so the absorbances recorded are comparable only as to location of maxima and minima. In Table 1 are the absorptions of the 21 cultures studied, giving only the wavelengths which reveal peaks, to conserve space. Detailed absorptions are in the thesis of TWAROG (1958). Reference to Table 1 reveals that F-958, F-358, F-8315, and F-10234 are quite similar with the maxima at 402—410 mμ and 476—489 mμ.

Strains F-100 and F-8366 absorbed strongly only at 402—410 mμ; strain F-4651 only at 418—420 mμ; strains F-48 and F-700 and F-8091 at 420—424 mμ as well as at 476—478 mμ. *F. aquatile*, F-700, regarded as a species of *Pseudomonas* by WEEKS (1955), is seen here to resemble some flavobacteria, but clearly differs from others. Figure 1 is an illustrative plot of the complete absorbance of F-958.

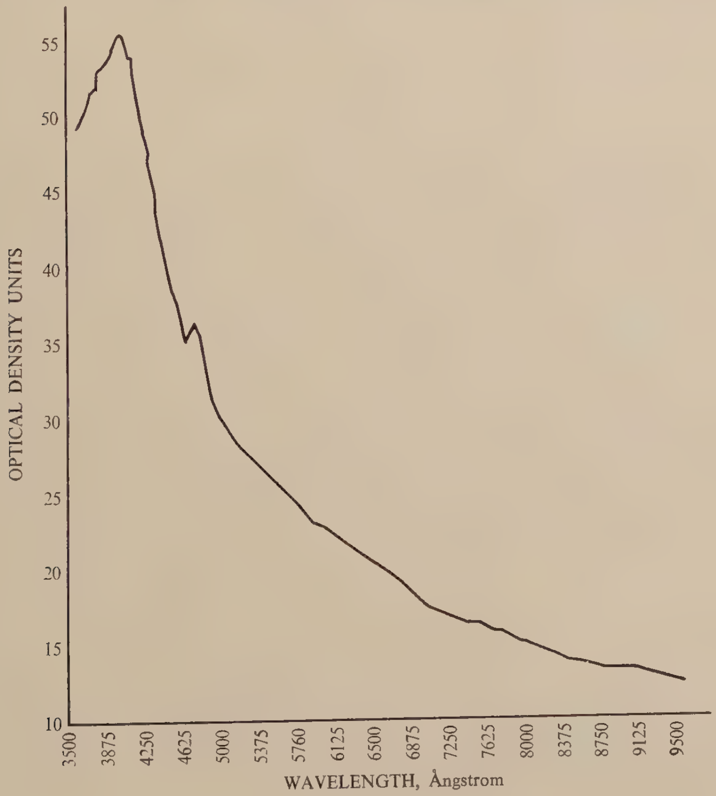

Fig. 1.

Reference to Table 1 also shows that the species of *Xanthomonas* studied were remarkably similar, in their absorbance at 400—406 mμ, except X-Bl which showed a peak only at 390—395 mμ and X-7381 which showed a peak at 450 mμ as well as at 400—406 mμ.

It is seen that the 2 species of *Corynebacterium* showed peaks at 400—404 mμ and at 476—480 mμ, which is quite similar to those of most of the flavobacteria studied.

It is evident that the generic differentiation by this procedure is most clear-cut for *Xanthomonas*, and falls far short of the great diagnostic value anticipated.

An extensive study was made of the absorbance of methanol extracts of 6 representative cultures, which revealed practically identical peaks, with a few clearly discernible others at intermediate wavelengths, probably owing to the incomplete extraction, or the masking effects of pigment of similar absorbances in the cellular suspensions. The use of clarified suspensions appears hence to be a feasible procedure for the study of biosynthesis of pigments, but to make a limited contribution to taxonomy.

Summary.

A study of the spectral properties of intracellular pigments in clarified cellular suspensions of 11 flavobacteria, 9 xanthomonads and 2 corynebacteria showed that the flavobacteria are most heterogeneous in their absorption maxima (390, 400—410, 420—424, 476—478 mμ). The corynebacteria absorbed strongly at 400—404 mμ, indicating some similarity to the flavobacteria. The xanthomonads generally absorbed strongly at 404—406 mμ. In these genera, the general absorption properties removed from the procedure the anticipated taxonomic application, although after establishing other features, such study might be of profit especially in the case of designation of *Xanthomonas*.

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SEROLOGICAL STUDY OF A PLEUROPNEUMONIA-LIKE ORGANISM AND AN ASSOCIATED *CORYNEBACTERIUM* SPECIES[1]

by

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and **JOHN E. FABER**

(Received March 6, 1961).

INTRODUCTION.

An increasing number of reports in the past several years has been concerned with the reversion of pleuropneumonia-like organisms (PPLO) to bacteria (McKAY and TAYLOR, 1954; WITTLER *et al.*, 1956; SMITH *et al.*, 1957; KELTON *et al.*, 1960; GENTRY, 1960; MORRISON, 1961). Only two of these reports have been supplemented by even limited serological studies of the organisms involved. WITTLER *et al.* (1956) reported agglutination of a PPLO strain by antiserum to a *Corynebacterium* sp. isolated from the same clinical specimen. This *Corynebacterium* was identical to the *Corynebacterium* resulting from the reversion of the PPLO. The above antiserum inhibited growth of the PPLO when incorporated into the medium on which the PPLO was cultivated. SMITH and ROTHBLATT (1960) studied the Campo strain of PPLO used by SMITH *et al.* (1957) in their "reversion" experiments. This PPLO was compared to two diphtheroids obtained by reversion of the PPLO strain. Agglutination and precipitation results, in addition to metabolic studies, led the authors to believe ". . . that the Campo PPLO may be a stable L form of a diphtheroid".

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The serological relationship between several classical L phase variants and their parent bacterial forms has long been recognized. KLIENEBERGER (1938) was the first to demonstrate the serological relationship between *S. moniliformis* and its L phase (L_1) by agglutination tests. DIENES *et al.* (1950) and SHARP and DIENES (1959) also demonstrated such a serological relationship between *Proteus* and its L phase variant. On the basis of these studies it has been commonly assumed that all L phase variants can be expected to exhibit a significant cross serological reaction with their respective bacterial parents, and further, that where no such cross reaction can be demonstrated, proof of a generic relationship remains incomplete.

Since the potential ability of a PPLO strain to revert to bacterial form is still a controversial subject, a more thorough serological investigation of a PPLO strain and its presumed bacterial derivative was considered desirable. For this investigation PPLO 4387 and *Corynebacterium* sp. (C4387) were employed. In addition to the data reported by WITTLER *et al.* (1956), information about these strains had been collected by other workers. KLIENEBERGER-NOBEL had examined PPLO 4387 and had determined that the strain was a typical human genital PPLO similar to other *Mycoplasma hominis* strains in her collection. Her conclusions were based on the results of morphological, cultural, and serological studies. Furthermore, she found no cross reactions by the complement fixation test between PPLO 4387 and *Corynebacterium* sp. C4387 (personal communication). MORRISON (1961) and MORRISON *et al.* (1961) reported that PPLO 4387 could be induced to revert to a *Corynebacterium* in cell-free media and concluded that the organism should be classified as an L phase variant rather than a species of *Mycoplasma*. The quantity and divergency of data already available on this PPLO and *Corynebacterium* made them a suitable pair of strains for further study.

MATERIALS AND METHODS.

Source of organisms. Three organisms were used in this study: PPLO 4387, *Corynebacterium* sp. (C4387), and *Staphylococcus albus* (S 4387). They were isolated concurrently from the urethral exudate of a patient with non-gonococcal urethritis by WITTLER *et al.* (1956). The *Staphylococcus albus*, though apparently unrelated to the PPLO and *Corynebacterium*, supplied factors that promoted the reversion of the PPLO to the *Corynebacterium*. It was included as a control in this study to detect possible non-specific cross reactions between the PPLO and other organisms. The organisms had been maintained in the frozen state at —55°C. between the original and present studies. The three strains were repurified by repeated single colony isolations for the current work. The PPLO was cultured in the absence of penicillin and all other substances known to induce the L phase of growth from bacteria.

Media. Bacto veal infusion broth (VIB) ¹) and Bacto veal infusion agar (VIA) ¹) were used as basal media for the growth of the PPLO and the *Corynebacterium*. Ascitic fluid was added to a final concentration of 20 % for the cultivation of PPLO, and rabbit serum was added to a final concentration of 10 % for the cultivation of the *Corynebacterium*. These media are designated hereafter as VIB-AF, VIA-AF, VIB-RS and VIA-RS respectively. Trypticase soy agar (TSA) ²) was used for the cultivation of the *Staphylococcus*.

Enrichment. The ascitic fluid was obtained from a single patient. Pooled normal rabbit serum was obtained from Cappel Laboratories, Inc., West Chester, Pennsylvania. Ascitic fluid and rabbit serum were sterilized by filtration through Seitz filter pads (APD 0.01 μ) and stored in the frozen state. Before use they were refiltered and heated at 60°C. for 30 min.

Buffered saline. Triethanolamine buffered saline (TBS) was used for all procedures requiring buffered saline including the complement fixation test.

PPLO antigen. PPLO were grown in 1 liter Erlenmeyer flasks containing 250 ml VIB-AF. Five ml of a 48 hr. tube culture of PPLO were used as an inoculum, and the flasks were incubated at 37°C. for 72 hr. Before harvesting, the flasks were examined by Gram stain and by plating on duplicate plates of VIA-AF. Any evidence of bacterial growth caused the flask or lot of antigen to be discarded. PPLO cultures were centrifuged at 30,000 × G for

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¹) Difco Laboratories, Detroit, Michigan.

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35 min. and washed twice with TBS. The final concentration was adjusted to match a number 5 McFarland nephelometer. This antigen was used for animal inoculation as well as for the complement fixation test. The antigen was frozen and stored without the addition of preservatives.

Bacterial antigens. The *Corynebacterium* was grown on VIA-RS plates. Twenty-four hr. growth was washed from the plates with TBS, and the suspension of growth from each plate was examined by Gram stain for contamination. Contaminated plates were discarded. The cells were heated at 60°C. for 15 min. and washed twice with TBS. The portion of washed resuspended cells used for animal inoculation was adjusted to contain the approximate equivalent of 10^{10} cells/ml. The remainder of the antigen was diluted to match a number 5 nephelometer and after titration and appropriate dilution was used as an antigen for the complement fixation test. Merthiolate was added to a final concentration of 1 : 10,000, and the antigens were refrigerated until used.

The *Staphylococcus* was grown on plates of TSA. Twenty-four hr. growth was washed from the plates and the suspensions examined for contamination as above. The cells were heated for 45 min. at 60°C. and washed twice in TBS. The antigen suspensions were prepared as for the *Corynebacterium*.

Lysed *Corynebacterium* and lysed *Staphylococcus* antigens were prepared by rupturing washed suspensions of cells (10^{10} cells/ml in 20 ml batches) in a 10 kc Raytheon sonic oscillator for 20—30 min. Further dilution and preservation were the same as for the whole cells.

Control antigens. Substances used as controls for the antigens were veal infusion broth, ascitic fluid, and veal infusion broth plus 20 % ascitic fluid. These media control antigens were prepared and sterilized in the same manner in which they were prepared for use as culture media. Merthiolate was added to a final concentration of 1 : 10,000 and the antigens were refrigerated. They were used undiluted for injection into the rabbits and for the complement fixation test.

Antisera. White male rabbits (7—10 lb.) were used for the production of antisera. Antigens were administered by injection in the marginal vein of the ear. Inoculations were made Monday, Wednesday and Friday of each week for three weeks. The following amounts were given: 0.5, 0.75, 1.0, 1.0, 1.5, 1.5, 1.5, 2.0, and 2.0 ml respectively for the 9 injections. Ten days after the last injection the animal was bled out by cardiac puncture. Several sera prepared for the earlier study (WITTLER *et al.*, 1956) were retested in this study. The methods reported for the preparation of the older sera differed slightly from the present methods.

Complement fixation test. The method used was a modification of that used by CHAFFEE *et al.* (1956). The reagent diluent was TBS and the total volume for the complement fixation test was 1.0 ml. The 50 % hemolytic unit of complement ($C'H_{50}$) was determined by precise spectrophotometric technic. Two tenths ml of serum were incubated with 0.2 ml of complement ($5C'H_{50}$) and 0.2 ml antigen for 16—18 hr. at 4°C. Sensitized erythrocytes (0.4 ml) were then added and the tubes incubated for 30 min. in a 37°C. water bath. The tests were read by visual comparison with standards that allowed estimation of hemolysis to the nearest 10 %. The titer of the serum is here defined as the reciprocal of the dilution giving a two plus reaction (40—60 % hemolysis). The mid-point of this range was chosen as an end point, and any serum that showed 50 % hemolysis or less was considered positive. Each lot of antigen was standardized by testing dilutions of the antigen against a positive homologous antiserum. That dilution of a particular lot of antigen giving the highest titer with the test antiserum was used thereafter.

RESULTS.

Complement fixation tests were first performed using the two media components VIB and AF, and the enriched media (VIB-AF) used for the growth of the PPLO. These substances and their homologous sera were used as controls for the antibacterial test system. Each complement fixation test was run two or more times using different lots of antigen each time. Since the rabbit antisera were usually anticomplementary at dilutions of less than 1 : 8, sera not reacting at a dilution of 1 : 8 were reported as negative. The paired pre-immune and immune sera from each rabbit were run simultaneously. It was found that pre-immune titers to the media components ranged from 8—32. The sera obtained from the rabbits after inoculation of the media components showed no rise in titer (Table 1). The same paired sera were tested against *Corynebacterium*, lysed *Corynebacterium*, PPLO, and *Staphylococcus* antigens. The pre-immune sera showed titers between 8 and 64 to all the organisms tested with no significant increase in titer to these organisms in the immune sera. It was, therefore, assumed that residual media or enrichment contained in the bacterial or PPLO suspensions would not account for the appearance of significant cross reactions between organisms.

TABLE 1.

Complement fixation tests of anti-media sera.

Sera		Antigens				
		Corynebacterium	Lysed *Corynebacterium*	PPLO	*Staphylococcus*	VIB-AF
VIB	imm.	0	64	16	0	8
	pre-imm.	16	64	32	0	8
AF	imm.	32	64	16	32	32
	pre-imm.	16	64	16	64	32
VIB-AF	imm.	32	64	16	32	16
	pre-imm.	32	32	16	8	8

During the course of the study the pre-immune sera of "normal" rabbits were tested against the various antigens. It was found that 50 % of the rabbits had titers of 8—32, 40 % had titers of 64, and 10 % had titers of 128 or more. These findings made it impractical to attempt to use only rabbits having negative pre-immune titers for the experiments. Instead, all rabbits having a pre-immune titer of 128 or more to any of the antigens were arbitrarily excluded. Furthermore, titers of 64 or less in those sera for which no pre-immune sera were available were assumed to have existed in the rabbit before immunization.

Since pre-immune titers to the various antigens were so prevalent, a small increase in titer to one of these antigens when a heterologous antigen was injected has no significance. The complement fixation test as used in this study is accurate only over a three tube range, that is, one dilution above and one dilution below the reported titer. Therefore, when two sera (immune and pre-immune) are compared, the titers must be separated by more than a two tube difference before a change in titer is reported.

Those sera produced earlier (Sera B, C, K, L, M, Q *cf.* Wittler *et al.*, 1956) were included in this study to determine whether the antigens had changed considerably during the several years of storage. Although this was the first time complement fixation titers were obtained for these sera, the titers were sufficiently high to indicate that the antigens had not changed appreciably during storage or passage.

Table 2 shows the results obtained when anti-*Corynebacterium* sera were tested against the various antigens. Following the criteria defined above only the titers to *Corynebacterium* and lysed *Corynebacterium* can be considered significant. All other titers either failed to show a three tube increase when pre-immune sera were available or did not exceed a titer of 64 when pre-immune sera were not available. Variation between individual rabbits is evident when the titers to *Corynebacterium* of sera F and A (titer of 256) are compared to the titers (512—1024) of other sera in this group.

TABLE 2.

Complement fixation tests of anti-*Corynebacterium* sera.

Sera		Antigens				
		Corynebacterium	Lysed *Corynebacterium*	PPLO	*Staphylococcus*	VIB-AF
C	imm.	2048	—	0	—	0
	pre-imm.	—	—	—	—	—
B	imm.	1024	1024	8	0	8
	pre-imm.	—	—	—	—	—
E	imm.	1024	1024	8	8	64
	pre-imm.	8	32	8	16	64
D	imm.	512	1024	0	0	8
	pre-imm.	—	—	—	—	—
F	imm.	256	512	16	16	32
	pre-imm.	16	32	32	8	16
A	imm.	256	256	8	0	16
	pre-imm.	16	32	8	0	16

— test not done.

Several antisera were produced to lysed *Corynebacterium*, but because of high pre-immune titers only one was included here. Again as with the anti-*Corynebacterium* sera (Table 2) the only titers that showed the necessary three tube rise were those to whole or lysed *Corynebacterium* antigens (Table 3).

TABLE 3.

Complement fixation tests of anti-lysed *Corynebacterium* serum.

Serum		Antigens				
		Coryne-bacterium	Lysed *Coryne-bacterium*	PPLO	*Staphylo-coccus*	VIB-AF
H	imm.	512	1024	0	0	16
	pre-imm.	0	16	0	0	8

Six anti-PPLO sera were tested. With one exception the only titers considered significant were those to the homologous PPLO antigen (Table 4). The one exception involved a three tube titer increase in serum N when tested against VIB-AF. On first inspection this might appear to be due to the inoculation of media into the rabbit along with the PPLO. This, however, was not supported by the control experiments and probably was the result of a non-specific response in this rabbit.

TABLE 4.

Complement fixation tests of anti-PPLO sera.

Sera		Antigens					
		Coryne-bacterium	Lysed *Coryne-bacterium*	PPLO	*Staphylo-coccus*	Lysed *Staphylo-coccus*	VIB-AF
L	imm.	0	16	2048	8	16	64
	pre-imm.	—	—	—	—	—	—
K	imm.	8	32	1024	32	32	32
	pre-imm.	—	—	—	—	—	—
M	imm.	0	32	512	0	0	16
	pre-imm.	—	—	—	—	—	—
N	imm.	32	128	1024	32	128	128
	pre-imm.	32	64	0	32	64	16
P	imm.	8	16	512	0	64	64
	pre-imm.	0	32	8	0	32	32
J	imm.	16	64	512	16	32	32
	pre-imm.	—	—	—	—	—	—

— test not done.

Two anti-*Staphylococcus* sera were included in this study as negative controls. Only the titers to the homologous antigen, *Staphylococcus*, were significant (Table 5).

TABLE 5.

Complement fixation tests of anti-*Staphylococcus* sera.

Sera		Antigens				
		Corynebacterium	Lysed *Corynebacterium*	PPLO	*Staphylococcus*	VIB-AF
Q	imm.	16	64	0	256	16
	pre-imm.	—	—	—	—	—
R	imm.	0	32	16	512	16
	pre-imm.	—	—	—	—	—

— test not done.

None of the sera in this study produced significant titers to other than its homologous antigens on the basis of the defined criteria. It may, therefore, be stated that the complement fixation test as used here did not demonstrate a serological cross reaction between the PPLO and the *Corynebacterium*.

Discussion.

The lack of cross reactions between the PPLO and *Corynebacterium* are in accord with the results obtained by Klieneberger-Nobel. However, in view of the results of Morrison (1961) and Morrison *et al.* (1961) who obtained reversion of the PPLO to *Corynebacterium* 15 times or more using several methods, the applicability of the complement fixation test to the solution of the problem is questionable.

Growth inhibition tests using the sera from both the earlier study and from this study indicate that a relationship between PPLO and *Corynebacterium* can be demonstrated by this method. The preliminary tests which have been carried out show unexplained variations which may be due to variation in the growth phase of the PPLO. Work is continuing on this phase of the investigation.

If the PPLO and *Corynebacterium* are indeed related, it is possible that such a relationship would not be detected by complement fixation tests. All antigens are not detectable using any one given serological test. It is necessary, therefore, to use several methods to examine the organisms. Even when this is done, if no common antigens are detected, the only conclusion that can be drawn is that using the given methods no serological cross reactions were observed. It is not evidence *per se* that the organisms are not related.

The L phase of *Proteus* can grow in two different colonial forms (Dienes and Weinberger, 1951). Minck and Kirn (1960) found that growth of the 3A type is inhibited by specific antisera while the 3B type is not. The two types also differ in their morphological and cultural characteristics. It has been observed that PPLO 4387 at times develops two colony types somewhat analogous to the 3A and 3B types of *Proteus*. The "3A" type is found when the culture is rapidly passed (24 hr.). This variation in colony types is believed to account for variations found in our growth inhibition tests. It is not known with certainty in what phase the organisms existed in the broth cultures used for the complement fixation antigens. The existence of the PPLO in one phase rather than the other may account for a lack of cross reaction in the complement fixation test.

Because of the above considerations the results of this study cannot be accepted as the sole and final evidence that PPLO 4387 and *Corynebacterium* C4387 are not related.

Acknowledgements.

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Summary.

The complement fixation test was employed to study the possible serological relationship between PPLO 4387 and *Corynebacterium* C4387. No cross reactions between the two organisms were demonstrated by the methods employed. The limitations of the complement fixation test in this study were discussed. Because of these limitations and the accumulated evidence from other types of studies, the authors concluded that the results obtained are not

necessarily proof of the absence of a relationship between the PPLO and the *Corynebacterium* sp.

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BRETTANOMYCES CUSTERSIANUS NOV. SPEC.

by

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The association of the genus *Brettanomyces* with the beer, wine and cider industries suggested that these yeasts might be recovered from other fermentation industries.

By means of the enrichment techniques described previously (van der Walt and van Kerken, 1961), the occurrence of the genus *Brettanomyces* in the Bantu beer industry has now been clearly established. From fifteen of twenty-four samples drawn from two Bantu beer breweries in the Witwatersrand area, and collected from sources such as drains, contaminated brewing equipment and floor surfaces, cultures of *Brett. intermedius* together with thirty strains of an undescribed species were recovered.

The following description of this new species, which has been named *Brett. custersianus*, is based on the standard methods of Wickerham (1951, 1957) and Lodder and Kreger-van Rij (1952).

Description.

Growth in malt extract: After 5 days at 25°C., the cells are short-oval, frequently ogive, long-oval, cylindrical to very much elongated, single, in pairs or short chains. Pseudomycelium is formed. The cells measure (1.2 — 3.8) × (2.5 — 15 — 27) μ. A sediment is produced. Islets or a thin pellicle are usually formed, but these may be absent. After 10 days at 15°C., a dry pellicle or islets are usually present. The pellicle may disintegrate and sink. Occasionally an incomplete ring is formed. In malt extract, as on malt agar, a characteristic aroma develops.

Streak culture on malt agar: After 8 days at 15°C., creamish-yellow, glistening, raised, not spreading, smooth or with some structure, verrucose or crispulate. Margin smooth. After 6 weeks at 15°C., light brown, glistening, not spreading, soft, smooth or with some structure or verrucose. Margin smooth, undulating, with some structure or lobiform.

Streak culture on malt agar with calcium carbonate: After 6 weeks at 15°C., creamish-brown, dull, raised, not spreading, verrucose or crispulate. Margin lobiform.

Sporulation: Not observed.

Slide cultures: Pseudomycelium is abundantly produced. It is not much branched and bears few or no blastospores. True mycelium is absent.

Fermentation:

Glucose	+	Trehalose	+ (latent)
Galactose	—	Lactose	—
Saccharose	—	Melibiose	—
Maltose	—	Raffinose	—
Melizitose	—		

Carbon assimilation: Glucose, trehalose, saccharose (weak), ethanol, glycerol, potassium D-gluconate (weak), DL-lactic acid and succinic acid are assimilated but not galactose, L-sorbose, maltose, cellobiose, lactose, melibiose, raffinose, melizitose, D-xylose, D-arabinose, L-arabinose, D-ribose, L-rhamnose, *i*-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, α-methyl-D-glucoside, salicin, citric acid and *i*-inositol.

Acid production from glucose (according to standard method): Positive.

Assimilation of ethylamine hydrochloride: Positive (slow).

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Absent.

Growth in osmotic pressure medium: Absent.

Growth in the absence of an extraneous vitamin source: Absent.

Growth at 37°C.: Positive.

DISCUSSION.

Ascospore formation was not observed in any of the thirty individual strains, or in any matings of these strains. However, it was likewise impossible to confirm ascospore-formation in any of the concomitantly recovered cultures of *Brett. intermedius* — a species in which ascospore formation was established (van der Walt and van Kerken, 1960). The sporulating ability of the cultures would therefore appear to have been affected by the particular environment from which these cultures were obtained. The creation of a form genus for the classification of species in which ascospore formation has not yet been verified does therefore not seem warranted.

Despite the apparent absence of ascospore-formation in the strains of the new species, inclusion in the genus *Brettanomyces* is justified on cultural, morphological and biochemical grounds. Like all other accepted species of the genus, the strains of *Brett. custersianus* are characterized by: their slow growth, the formation of ogive or sometimes somewhat apiculate cells, the production of the characteristic "*Brettanomyces* aroma", development at 37°C., and their inability to develop in media of moderately high osmotic pressure, the inefficient terminal respiration of glucose resulting in the accumulation of excessive amounts of acetic acid, the utilization of ethylamine as nitrogen source, a marked vitamin dependence requiring both biotin and thiamine and an insensitivity towards actidione. Like other species of the genus, *Brett. custersianus* also shows a "negative Pasteur effect" (*cf.* Wikén and Pfennig, 1959).

Although the fundamental characteristics of the genus were described by Custers (1940), it has not been possible to formulate a phylogenetic line for *Brettanomyces* because of the apparent absence of less advanced members of the genus. The existence of more primitive members has now been established with the recovery of *Brett. custersianus* as is evident from its reduced fermentative and assimilatory abilities and from the comparatively primitive manner of its pseudomycelium formation.

As the species is also markedly filamentous, it indicates that development within the genus proceeded along at least two lines. The first and more primitive line includes the more or less filamentous species *Brett. custersianus* and *Brett. anomalus*. The second line of development is represented by the species *Brett. bruxellensis*, *Brett. intermedius* and *Brett. schanderlii*. The position of *Brett. claussenii*, however, is at present somewhat uncertain. Although this species represents the most advanced member biochemically, fermenting at least nine sugars, it lacks the ability to form pseudomycelium — a characteristic common to all hitherto known members of the genus.

The inability of *Brett. custersianus* to utilize nitrate confirms the views of CUSTERS (1940), DIDDENS and LODDER (1942), WICKERHAM (1952) and LODDER and KREGER-VAN RIJ (1952) that nitrate assimilation is not a fundamental characteristic of the genus. Furthermore, the recovery of a second nitrate-negative species supports the separation of *Brett. schanderlii* from *Brett. intermedius* — two species which have been found to differ mainly in their utilization of nitrate.

The new species has been named in honour of Dr. M. TH. J. CUSTERS in recognition of his pioneering studies on the genus *Brettanomyces* as a taxonomic entity. Cultures of *Brett. custersianus* have been deposited in the yeast collection of the Centraal Bureau voor Schimmelcultures in Delft.

Brettanomyces custersianus nov. spec.

In extracto malti (post dies 5, 25°C.) cellulae subovoidiae, 'ogive' modo apicatae, longovoidiae, cylindricaeque, singulae, binae aut in catenatae. Cellularum pseudomycelii longiores formantur. Dimensiones cellularum sunt (1.2 — 3.8) × (2.5 — 15 — 27) μ. Cellulae lentissime crescunt. Sedimentum. Insulae aut pellicula tenuis, non nitida formantur. Aliquando pellicula cadet ad fundum. Plerumque anulus imperfectus formatur. In extracto malti et in agar malti odor insignitus.

In agar malti (post sex septimas, 15°C.) cultura subfusca, nitida, acclivis, non se sternens, glabra, cum structura, verrucosa aut crispulata. Margine glabro, structuris aut lobiformo.

In agar malti cum calcium carbonicum (post sex septimas, 15°C.) cultura flavifusca, non nitida, acclivis, non se sternens, crispulata aut verrucosa. Margine lobiformo.

Pseudomycelium abundat; parum ramosum. Cellulae pseudomycelii plerumque sine blastosporae. Mycelium verum nullum.

Glucosum et trehalosum (tardum) fermentantur at non galactosum, saccharosum, maltosum, lactosum, melibiosum, cellobiosum, raffinosum nec melizitosum.

Glucosum, trehalosum, saccharosum (exiguum), ethanolum, glycerolum, kalium D-gluconicum (exiguum), DL-acidum lacticum et acidum succinicum assimilantur at non galactosum, L-sorbosum, maltosum, cellobiosum, lactosum, melibiosum, raffinosum, melizitosum, D-xylosum, D-arabinosum, L-arabinosum, D-ribosum, L-rhamnosum, *i*-erythritolum, adonitolum, dulcitolum, D-mannitolum, D-sorbitolum, α-methyl-D-glucosidum, salicinum, acidum citricum nec *i*-inositolum.

In conditione aerobia vehementer acidum aceticum de gluconibus formatur.

Ethylaminum hydrochloricum assimilatur at non kalium nitricum nec natrium nitrosum.

Arbutinum non finditur.

Necessariae ad crescentiam sunt vitaminae externae.

Crescentia fieri potest in 37°C.

Acquisitum ex aedificiis ad cerevisiam Afram coquendam extructis.

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ON THE FORMATION OF ADIPOCERE FROM FATS

CONTRIBUTION TO THE MICROBIOLOGY OF SYSTEMS CONTAINING TWO LIQUID PHASES

by

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with the technical assistance of Miss M. S. DAUVILLIER and Mr. W. B. ROMAN

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This study originated from a request of the Minister of Social Affairs and Health Care in the Netherlands to the Chairman of the Council of Health, Dr. J. Wester, for an investigation of the circumstances which lead to the formation of adipocere in interred bodies. In 1955, Dr. Wester nominated a Committee which on June 30th, 1961 presented a Report on the incomplete decomposition of interred bodies.

During the evacuation of the graves in a cemetery in one of the larger cities in this country – a measure which according to Dutch law may not be taken before 10 years have passed since interment – the Committee established that more than 50% of the exhumed human remains were not, or not completely decomposed. The fact that the dead bodies for the greater part had been converted into "adipocere images", a state of affairs involving ethical as well as social-economical consequences, convinced the Government of the desirability of a closer investigation of the processes which occurred in the interred bodies.

The microbiological aspects of the problem were studied by the present author as a member of the above-mentioned Committee and the following article constitutes Appendix IV of its Report.

Adipocere (latin: *adeps* = fat; *cera* = wax) is the term coined by Fourcroy for a conversion product which may be formed during incomplete decomposition of human and animal remains. The first to draw attention to this phenomenon was R. BOYLE. The early literature as well as more recent knowledge on the subject has been exhaustively reviewed by MANT (1957). A condensed description of the main lines of development, adapted from MANT's article is presented in the following:

The first authentic description of adipocere was presented by FOURCROY (1789). His investigations were carried out during the second half of the 18th century, when numerous human remains were exhumed in the Paris cemetery "Cimetière des Innocents". In his publication he gave an excellent description of adipocere: a homogeneous, grey-white, friable substance without offensive odour. In the more recently buried body (3–5 yrs) pigmented muscle groups were still recognizable although surrounded by adipocere. In the later groups (30–40 yrs) muscle fibers, *etc.* had disappeared. He observed that adipocere formed in the fatty regions such as the cheeks and breasts.

During exhumations performed in the Netherlands also the neck, the abdominal outer layer and the thighs appeared to be converted into adipocere. In some cases this substance was deposited outside of the body, between and along the legs, near the neck and shoulders.

The internal viscera were not always recognizable but were often found in a greatly shrunken state. The freshly formed adipocere was rather soft and wet, whereas the older material was dry and brittle. The grave-diggers at the cemetery asserted that 3 years were necessary before the body fats became changed into this substance.

FOURCROY carried out some quantitative tests on adipocere, which he believed to be an animal soap due to the combination of fat with ammonia. After this pioneer work, numerous observations were made by others but at present most of the interpretations given are no longer acceptable.

The current views in the earlier part of the 19th century were briefly that adipocere was an animal soap arising out of the decomposition of body fat. Analyses were made during this period by various chemists whose work was summarized by GMELIN (1864); adipocere was found to consist for some 97% of fatty acids, including palmitic and stearic. More recently RUTTAN (1916) examined both human and animal adipocere and found that human and pig adipocere were similar. He analysed adipocere and found that it consisted essentially of fatty acids and that the process was complete when the last trace of oleic acid had disappeared.

The chemical composition of pig adipocere as given by Marshall and Ruttan (1917) is as follows:

	Per Cent
Palmitic acid	67.52
Stearic acid	3.3
Oleic acid	5.24
i-Hydroxystearic acid	9.48
o-Hydroxystearic acid	6.32
Stearin and palmitin	1.21
Olein	0.16
Unsaponified matter	0.87
Calcium soaps	4.41
Protein	0.665
Ash	0.578
Humus and undetermined substances	0.247

Ruttan considered adipocere a product of the hydrolysis of fat by water under conditions where the time factor and the concentration of the water are almost unlimited and where the glycerol formed during the hydrolysis is rapidly removed. He held the opinion that bacteria and enzymes played a secondary role only.

The following years produced little new. The factors generally accepted as being essential for, or accelerating, adipocere formation, were an excess of moisture, warmth, the presence of skin and subcutaneous tissues and, according to Glaister (1950) a relative diminution of air and micro-organisms. Mant (1953) himself contributed interesting observations made during the exhumations of several hundreds of bodies in North-western Europe during the years 1945–1948 and in his review arrives at the opinion that in the early stages of decomposition bacteria are essential to adipocere formation.

The observation that adipocere had not been formed in bodies of persons, buried in the period shortly before and during the "starvation winter" of 1944–1945, but was actually present in bodies from the later part of 1945, after the nutritional condition of the Dutch population had grown normal again, suggested also to the present author that adipocere is formed from fats and not from protein. Moreover, theoretical considerations make it hardly acceptable that proteins with their peptide bonds between amino acids and their numerous side chains can be converted into fatty acids, the investigations cited above having shown that adipocere – which in contradistinction to the etymology of the term contains neither fat nor wax – consists essentially of higher saturated fatty acids, mostly palmitic acid, hence of long chains of -CH_2-CH_2-groups, preceded by a CH_3-group and ending in a carboxyl group -COOH.

From the fact that adipocere was mainly found in the coffins, buried at greater depth, *viz.*, in the 3rd and 4th from the top, it could be concluded that a low supply of oxygen would promote the formation of this substance.

The above data suggested experiments in which bacteria were induced to attack fats under virtually anaerobic conditions. As human fat is rather hard to obtain, these experiments were initially carried out with olive oil, which is also a liquid, unsaturated fat. Since, obviously, olive oil alone cannot be attacked by bacteria, water had to be added. Accordingly, we employed a nutrient medium, consisting of a layer of oil floating on a water phase. The water phase was provided with nutritional salts, *i.e.* dipotassiumphosphate and ammoniumsulphate as sources of potassium and phosphor, and of nitrogen and sulphur respectively. Some calciumcarbonate was added; tapwater was used in order to supply trace elements. After inoculation with garden soil it could be expected that in accordance with the elective principle of WINOGRADSKY and BEIJERINCK those bacteria would come to the fore, which possessed the property to attack olive oil as sole source of carbon and energy. Since adipocere formation occurs under virtually anaerobic conditions, the initial experiments were carried out in vessels practically entirely shut off from outer air.

EXPERIMENTAL.

A. Experiments with media, containing in the water phase inorganic salts only.

1. The standard test.

Initially erlenmeyer flasks of 2 l were employed with 1 l of a tapwater solution containing 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$ and 0.2% $CaCO_3$. Then 5% olive oil was introduced which formed a layer of 3 to 4 mm thickness on the water phase. The medium was inoculated with garden soil.

This can be performed by bringing the soil on top of the oil and shaking the inoculum through the medium. It is better still – in order to reduce the risk of moulding – to inoculate the water phase first and add the oil afterwards. Later experiments showed that an extremely diluted suspension of garden soil sufficed to bring about the process concerned, although more slowly than after inoculation with larger quantities of soil, such as 1 or 2 g. Also soil, pasteurized at 80° C., appeared to be capable to induce the conversion in the long run.

The flask was then closed with a rubber stopper, provided with a thin, cotton-plugged glass tube (inner core about 1 mm). By this means virtually anaerobic conditions could establish themselves while any gas formed could freely escape.

After 2 months' incubation at 25° C., the fairly thin oil layer appeared to be replaced by a crust of 10 to 12 mm, *i.e.* 3 to 4 times thicker than the oil layer. This crust was greyish white, sometimes greyish pink, of a corklike porosity, floating on the water. Its composition will be discussed in a later paragraph. When after removal of the stopper a burning candle was lowered into the flask, the flame was already extinguished near the neck of the flask, thus indicating practically complete disappearance of oxygen. In a cotton-plugged flask the candle keeps burning and in that case the crust formation proved to be much retarded.

A tube with baryta water, when introduced into the flask, would quickly show a milky white turbidity, thus indicating the presence of an appreciable amount of carbon dioxide. Furthermore, hydrogen sulphide could be demonstrated with lead acetate (also organoleptically) and methylene blue would be reduced, indicating a low redox potential of the water phase. All this demonstrated the presence of virtually anaerobic conditions in the flask.

In those cases where a sound dry crust had replaced the oil phase, the microflora appeared to consist of rods. Sometimes vibrio's, probably of *Desulfovibrio desulfuricans*, which reduces sulphate to hydrogen sulphide, could be recognized among them and perhaps also some methane fermentation had occurred. Nevertheless in a number of cases the surface of the crust would be covered with a multicoloured mouldy layer; this generally occurred when after inoculation anaerobic conditions had been established by introducing CO_2 from a cylinder, which obviously must have led to a lowering of the pH, rendering conditions more favourable for the development of moulds. Also in these cases H_2S as well as CO_2 could be demonstrated in the flasks.

Gaschromatographical analysis, performed in the Unilever Research Laboratory at Vlaardingen[1]), showed that the reaction product consisted mainly of palmitic acid (Table 1). This obviously had been formed from the oleic acid, which linked to glycerol, is present to over 80% in olive oil. Thus this acid must have lost two carbon atoms and have been hardened by the uptake of two hydrogen atoms. Furthermore it is clear that also a new fatty acid had been formed during the microbial process, *i.e.* hydroxystearic acid, which does not occur in olive oil.

TABLE 1.

Relative abundance of fatty acids present in olive oil and in the microbial reaction product, determined by means of gaschromatography and expressed in % of weight.

Fatty acids		Olive oil	Reaction product
C_{12}	lauric acid	0–1	—
C_{14}	myristic acid	0–2	2
C_{16}	palmitic acid	10–12	53.5
C_{18}	stearic acid	2–3.5	5
$C_{16}{}^{1})$	palmitoleic acid	1	—
$C_{18}{}^{1})$	oleic acid	70–83	18.5
$C_{18}{}^{2})$	linolic acid	7–13	trace
$C_{20}{}^{1})$		0–2.5	—
C_{20-24}			2
"C_x"	hydroxystearic acid		19
			100%

[1]) Contains one double bond. [2]) Contains two double bonds.

The analyses were performed by the Unilever Research Laboratory, Vlaardingen.

The experimental results described above can be explained by accepting the occurrence of reactions, which, according to present knowledge (*cf.* review by LYNEN, 1955) are commonly encountered in lipid metabolism. If we omit for the sake of simplicity the participation of coenzymes from our considerations, the dehydrogenation with concomitant shortening of the oleic acid molecule can be represented as follows, essentially along the lines of KNOOP's β-oxidation theory:

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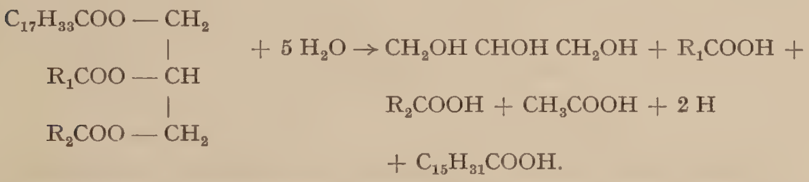
[1]) I am greatly indebted to Dr. J. P. K. VAN DER STEUR for his readiness to have his scientific staff collaborate in these investigations.

$$
\begin{array}{l}
CH_3(CH_2)_7CH = CH(CH_2)_5CH_2\ CH_2COOH \\
\text{oleic acid} \qquad\qquad\quad \downarrow \\
CH_3(CH_2)_7CH = CH(CH_2)_5CH = CHCOOH + 2\ H \\
\qquad\qquad\qquad\qquad\quad \downarrow + H_2O \\
CH_3(CH_2)_7CH = CH(CH_2)_5CHOH\ CH_2COOH \\
\qquad\qquad\qquad\qquad\quad \downarrow \\
CH_3(CH_2)_7CH = CH(CH_2)_5CO\ CH_2COOH + 2\ H \\
\qquad\qquad\qquad\qquad\quad \downarrow + H_2O \\
CH_3(CH_2)_7CH = CH(CH_2)_5COOH + CH_3COOH \\
\text{palmitoleic acid} \qquad\quad \downarrow + 2\ H \qquad\quad \text{acetic acid} \\
CH_3(CH_2)_7CH_2 - CH_2(CH_2)_5COOH \\
\text{palmitic acid}
\end{array}
$$

The last reaction step, the hydrogenation of the double bond, could also take place directly in the oleic acid or/and in any of the other intermediates. The fact that some hydroxystearic acid was found in the analysis might indicate that oleic acid is first hydrogenated to stearic acid before further dehydrogenation occurs. Irrespective of the level at which this hydrogenation takes place, the overall transformation of one oleic acid moiety in the fat originally present can be represented as follows:



$$
\begin{array}{l}
C_{17}H_{33}COO - CH_2 \\
\qquad\qquad\qquad | \qquad + 5\ H_2O \rightarrow CH_2OH\ CHOH\ CH_2OH + R_1COOH + \\
\quad R_1COO - CH \\
\qquad\qquad\qquad | \qquad\qquad\qquad R_2COOH + CH_3COOH + 2\ H \\
\quad R_2COO - CH_2 \\
\qquad\qquad\qquad\qquad\qquad\qquad + C_{15}H_{31}COOH.
\end{array}
$$

In this transformation, apart from palmitic acid, glycerol and acetic acid are formed which, together with the excess of hydrogen, are available for further microbial metabolic processes.

In order to confirm the formation of palmitic acid from oleic acid the above experiment was repeated with the fat triolein¹), put at our disposal by the courtesy of the Unilever Research Laboratory. This fat consists exclusively of the glycerol ester of oleic acid and hence contains none but C_{18}-fatty acid. If, therefore, the microbial reaction product would be found to contain lower fatty acids *e.g.* the saturated C_{16}-fatty acid (palmitic acid), proof would be furnished that an unsaturated C_{18}-acid may be shortened to a C_{16}-acid and simultaneously hardened to a saturated fatty acid. The following analysis demonstrates that microbes do possess the capacity of performing this reaction.

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¹) This experiment was suggested to us by Dr. J. BOLDINGH, Director of the Unilever Research Laboratory.

TABLE 2.

Gaschromatographic analysis of microbial conversion product of triolein. Fatty acid quantities are expressed in % of weight. Separate analyses were made of the ester fraction of the product and of the free fatty acids in the product.

Fatty acids	Amounts of fatty acids present		
	in ester fraction (4%)	as free acid (96%)	in total (100%)
C_{14}	—	1.0	1.0
C_{16}	5.0	15.0	14.6
C_{18}[1]	73.5	84.0	83.6
C_{20}[1]	21.5	—	0.8

[1]) contains one double bond.

The analyses were performed by the Unilever Research Laboratory, Vlaardingen.

In view of the aim of the investigation, repetition of the earlier experiments with human fat was obviously desirable. This fat was obtained from operatively removed tissue. It is partly liquid at room temperature; the method of the standard test was followed. After 2 months at 25° C. also here the oil phase appeared to be converted into a 3 or 4 times thicker crusty layer. Gaschromatographic analysis of this substance yielded the results presented in Table 3 (p. 345).

In close agreement with the data obtained in the investigation of the olive oil, we note that the content of (saturated) C_{16}-fatty acid (palmitic acid) has greatly increased at the expense of a decrease of unsaturated C_{18}-fatty acid (oleic acid).

Furthermore, the composition of the product obtained closely resembles that of "natural" adipocere samples, analysed by the Unilever Research Laboratorium (Table 4, p. 346). This makes it very probable (and it will become still more evident later on), that adipocere is generated by microbial conversions.

The fact that fatty acids, formed in the flasks, were not broken down any further, prompted a closer study of the bacterial degradation of fatty acids, in the first place under aerobic conditions.

In an earlier investigation (DEN DOOREN DE JONG, 1926, pp. 60-61) a number of aerobic and facultative anaerobic bacteria had been streaked on a solid medium which contained a fatty acid as sole source of carbon. Table 5 shows that saturated fatty acids become increasingly resistent to bacterial attack as the number of

TABLE 3.

Gaschromatographic analysis of fatty acid composition of human fat and its microbial conversion product. Fatty acid quantities are expressed in % of weight.

Fatty acids	Human fat	Reaction product
Saturated fatty acids:		
C_{12}	2.0%	2.0%
C_{14}	6.0%	7.0%
C_{15} and C_{15} branched	0.5%	0.5%
C_{16}	26.0%	53.0%
C_{17} and C_{17} branched	1.0%	1.0%
C_{18}	7.5%	9.0%
C_{20}	1.5%	1.5%
Unsaturated fatty acids:		
C_{16}¹)	5.0%	2.0%
C_{18}¹) and C_{18}²)	48.0%	22.0%
C_{20}¹)	2.0%	2.0%
C_{20}³)	0.5%	0.5%
	100.0%	100.0%

¹) contains one double bond. ²) contains two double bonds. ³) contains more than two double bonds.

The analyses were performed by the Unilever Research Laboratory, Vlaardingen.

C atoms increases. The unsaturated fatty acids, *viz.*, undecylenic and oleic acid, appear however, thanks to the double bond, more suitable for dissimilation. It may be concluded from Table 5 (p. 347) that the conversion of unsaturated fatty acids into saturated ones makes the latter more difficult for microbes to attack.

It appeared later, however, that some species of the genus *Mycobacterium*, *e.g. Myc. phlei*, *Myc. opacum*, *Myc. rubrum*, *Myc. globerulum* and *Myc. lacticola*, when streaked on similar culture plates, were able to dissimilate even palmitic and stearic acid.

Saturated higher fatty acids appeared nevertheless not to be attacked by bacteria, when suspended in the described liquid mineral medium and only under special conditions degradation by moulds occurred. On a solid medium, however, moulding may occur readily. Further investigation with a number of pure cultures of fungi showed, however, that only a low percentage was able to develop on tubes with the inorganic medium and 0.5% palmitic or stearic acid. The fungal growth sometimes occurring in the flasks inoculated with garden soil, has thus to be ascribed to the energy

TABLE 4.

Chemical and gaschromatographical analysis of two different samples of adipocere and one sample of freshly collected human fat.

Analysis of untreated samples	Sample		
	A	B	C
acid value	208	210	1.2
phosphorus	$< 0.003\%$	$< 0.003\%$	$< 0.003\%$
carotenoids	not detectable	not detectable	not detectable
unsaponifiable matter	0.80%	0.85%	0.64%
Analysis after saponification and removal of glycerol			
acid value	214	214	209
iodine number	13.8	12.6	73.5
Relative abundance of fatty acids in % of weight			
C_{12}	trace	trace	2.0
C_{14}	10.5	8.5	5.5
C_{15} (?)	1.0	1.5	—
C_{16}1)	—	—	5.0
C_{16}	67.5	73.0	28.5
C_{18}1)	7.5	5.0	55.5
C_{18}	6.0	8.5	3.0
C_{20}	7.5	3.5	0.5

1) contains one double bond.

Sample A was taken from an 85 years old body, sample B from a 15 years old one. Sample C represents fresh human fat.

The analyses were performed by the Unilever Research Laboratory, Vlaardingen.

producing dissimilation of unattacked olive oil, which along with oxygen, is initially still present in the flasks and will furnish excellent food for moulds.

When flasks, completely filled with either one of the mentioned fatty acid containing media were inoculated with garden soil which contains a world of bacteria as well as of moulds no growth occurred even after a long period of incubation. Under strictly anaerobic conditions the higher saturated fatty acids are apparently completely unassailable by microbes. This explains why adipocere bodies, provided they have been buried deep in the earth, will remain conserved indefinitely.

When particles of a crust, formed in a flask where the olive oil has been converted into saturated fatty acid without any moulding,

TABLE 5.

Dissimilation of fatty acids by various bacteria.

Tapwater agar with 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 1% $CaCO_3$ and 0.5% of the following acids:	*Bac. vulgatus*	*Bac. mycoides*	*Bac. polymyxa*	*B. aerogenes*	*B. coli*	*B. prodigiosum*	*B. herbicola*	*B. vulgare*	*Myc. phlei*	*Microc. albus*	*Sarc. lutea*	*Ps. fluorescens*	*Sp. tenue*
Formic acid	—	—	—	—	—	—	—	(—)	—	—	—	(—)	—
Acetic acid	(—)	(—)	—	+	(—)	(+)	(—)	(—)	+	—	+	+	(—)
Propionic acid	(—)	(—)	—	—	—	—	(—)	+	+	—	+	+	(—)
Butyric acid	(—)	(—)	—	(—)	—	—	—	(—)	+	—	+	(+)	(—)
Isobutyric acid	(—)	(—)	—	—	—	—	—	(—)	+	—	+	(+)	(—)
Valerianic acid	(—)	(—)	—	—	—	—	—	(—)	+	—	+	—	(—)
Caproic acid	(—)	(—)	—	(—)	—	+	—	(+)	+	—	+	+	(—)
Heptylic acid	(—)	—	—	—	—	(+)	—	(+)	+	—	+	+	—
Caprylic acid	—	—	—	—	—	+	—	—	+	—	+	+	—
Nonylic acid	—	—	—	—	—	+	—	—	(+)	—	+	+	—
Capric acid	—	(—)	—	—	—	+	—	—	(+)	—	(—)	+	—
Lauric acid	—	—	—	—	—	—	—	—	—	(—)	—	—	—
Palmitic acid	—	—	—	—	—	—	—	—	—	—	—	—	—
Stearic acid	—	—	—	—	—	—	—	—	—	(—)	—	—	—
Acrylic acid	—	—	—	(—)	—	—	—	—	—	—	—	—	—
α-crotonic acid	—	—	—	—	—	—	—	—	+	—	+	—	(—)
Undecylenic acid	—	—	—	—	—	—	—	(—)	(+)	—	(+)	(—)	—
Oleic acid	(—)	(—)	—	—	—	(+)	—	—	(+)	—	(+)	(+)	—
Elaidic acid	—	—	—	—	—	—	—	—	(—)	—	—	—	—

— = no growth; (—) = very weak growth; (+) = moderate growth; + = good or excellent growth. This table is reproduced from an earlier publication (DEN DOOREN DE JONG, 1926), which contains more detailed information on experimental technique and the bacterial strains investigated.

are streaked on meat broth agar, colonies of *Pseudomonas aeruginosa* appear. On plates containing olive oil these appeared to form a large part of the lipolytic flora. Apparently the lipolytic organisms from the garden soil had split the olive oil and anaerobic or facultatively anaerobic bacteria had mineralized the glycerol set free. When the conditions above the liquid had grown sufficiently anaerobic, an anaerobic or facultatively anaerobic flora had caused the oxydo-reduction of the fatty acids liberated. The oleic acid for instance would have lost two CH_2-groups and simultaneously have been hardened or hydrogenated to palmitic acid.

Up till now we have not succeeded in isolating from such cultures strains which were able to convert olive oil into crusts of fatty acids.

Transfers, however, were successful when a flask with the standard medium was inoculated with a crust particle containing the complete "bacterial team". By this means the inoculum would develop in most cases a crusty layer covering the whole of the liquid surface, the olive oil being converted into fatty acid. Inoculation with material from the water phase always remained negative.

2. Use of other inocula.

When instead of garden soil sandy soil from a graveyard was used as inoculum, crust formation could be achieved as well. Much more remarkable was the fact that this could even be brought about by faeces. Crust formation, however was then much delayed and became apparent after as much as 8 months. This may be explained by the fact that the main bacterial species in the faeces, *Escherichia coli*, is unable to bring about this conversion in a mineral medium. This has to be achieved by an accompanying flora, most probably acting as a team.

3. Variations in the composition of the standard medium and in the incubation temperature.

In order to speed up the creation of anaerobic or semi-anaerobic conditions in the flasks, 0.5% glucose or 0.5% glycerol was added to the standard medium (see p. 340). After inoculation with garden soil the flasks were kept incubated at 25° C. for 2 months. The conversion into fatty acids as indicated by crust formation appeared to be inhibited. In the flask with extra glycerol moulding was more pronounced. In the flask with extra glucose only in the long run the oil grew somewhat crusty.

Subsequently experiments were carried out in which olive oil was replaced by its main components, oleic acid (5%) and glycerol (0.5%). Also in this case a crust of fatty acids was formed in the long run which upon gaschromatographic analysis was found to consist of saturated fatty acids, including much palmitic acid.

Thus direct hardening of the oleic acid had been demonstrated. Better results were obtained when 0.5% glucose was substituted for glycerol; the crust formation markedly increased.

Since in all cases where a distinct crust formation had set in carbon dioxide had accumulated and hydrogen sulphide had been encountered, some sodium sulphide was added to the medium and the flask was filled with carbon dioxide from a cylinder. Also in this case after 2 months' incubation at 25° C. the result appeared to be unfavourable. Crust formation was delayed over a period of many months and the development of moulds had been stimulated, probably by the increase of hydrogen ion concentration.

For the same reason substitution of KH_2PO_4 for K_2HPO_4 appeared to inhibit the formation of fatty acid. The same result was obtained when KNO_3 was substituted for $(NH_4)_2SO_4$. Evidently nitrate is inhibitory for the development of a favourable redox potential.

Increase of the concentration of $(NH_4)_2SO_4$ up to 0.5 or 1% was inhibitory for crust formation. When a N source was omitted altogether, *Azotobacter* came to the fore and slight crust formation took place.

Summarizing, we may conclude that the original composition of the water phase as is given on p. 340 appears to be the most suitable one for the induction of the process in a mineral medium.

Further investigation was needed to decide whether the thickness of the oily layer of 3 mm used in all previous experiments was the most suitable one for the formation of saturated fatty acids. This factor was varied by adding oil in increasing amounts of 2.5, 5, 10 and 25%, the height of the layer thus varying from 1.5 to 3.5, 10 and 21 mm. After 2 to 3 months' incubation the oil in the flask with 2.5% appeared to be completely converted into fatty acid, and that in the flask with 5% only partly; with 10% a very thin grey layer could be noted at the interface between the water phase and the overlaying oil, which had turned turbid; with 25% the interface was still less marked and the oil possessed a milky turbidity (fig. 1).

Apparently the formation of fatty acid was mostly favoured by 2.5%; the standard amount of 5% still gave good results, but the higher quantities proved unsuitable for the formation of fatty acids. Evidently the aerobic lipolytic bacteria, localised at the oil-water interface, do not obtain enough oxygen under these conditions for active multiplication. As a result, the oil remains unattacked by anaerobic (or facultatively anaerobic) bacteria which normally perform the hardening of the unsaturated fatty acids, and crust formation fails to occur.

As completely shutting off from the outer air inhibits the formation of fatty acids, it appeared desirable to study the action of a

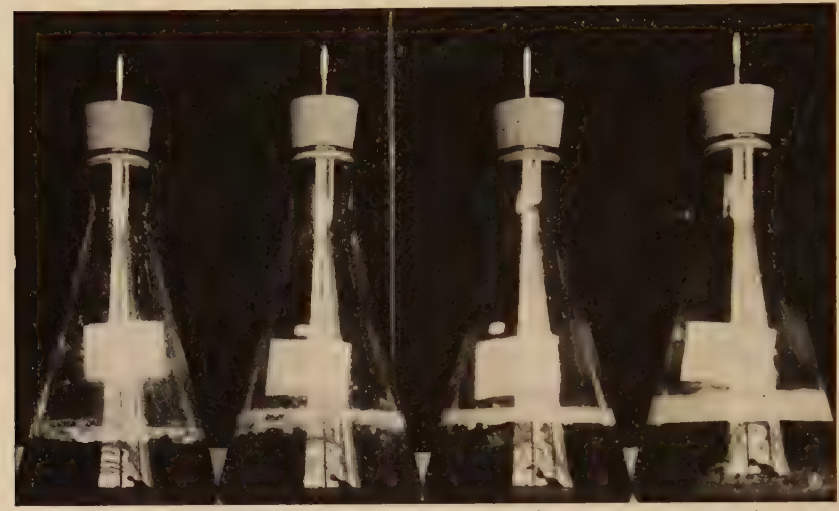

Fig. 1. Flasks with mineral medium and different quantities of olive oil after incubation at 25° C. for three months. The flasks contained originally 2.5, 5, 10 and 25% of olive oil respectively (1, 2, 3, 4) and garden soil as inoculum. In flasks (1) and (2) complete conversion of the oil occurred; flask (3) showed partial conversion and flask (4) none.

strong aeration on this process. The already cited fact, that in cotton-plugged erlenmeyers the conversion into fatty acids is delayed, had pointed to an inhibitory action. For a complete aeration of the oil surface, Petri dishes with a diameter of 20 cm – possessing the surface of the oil in the flasks – were half filled with the standard medium. After inoculation with garden soil 120 ml olive oil was poured on top, yielding a layer of about 4 mm. The lid was supported in such a way that air could enter the dish. As a control a stoppered erlenmeyer with a corresponding layer of oil was inoculated with the same garden soil. After some months' incubation a thick crust had been formed in the flask. In the Petri dish practically no crust formation could be observed. The oil had grown turbid and slimy, but any distinct formation of fatty acid or of moulding was lacking. Methylene blue added below the oil was not or very slowly decolorized in contrast to the rapid reduction observed in the flasks (see p. 341). Curiously enough, on the bottom of the dish, granular white or yellow-white, and sometimes orange-brown globular "colonies" had formed with a diameter of about 4 mm. Shaking loosened them from the bottom and made them float beneath the oil layer.

When picked up they showed a hard, granular surface. Microscopically the light-yellow particles appeared to contain many curved rods, the orange-brown ones broader or longer rods. In all probability they had arisen from oil drops deposited on the bottom of the dish and then converted into saturated fatty acids. Apparently the conditions at the bottom of the dish were more favourable for this process than those at the surface.

The process takes quite another course when the oil layer in the Petri dishes is thinner than 2 mm and hardly covers the water phase. Already after 3 weeks strong moulding of the oil occurs and within a short period of time the oil is mineralized.

Again a different course is noted when a similar, thin, oil layer is introduced on the water layer of an erlenmeyer closed with a perforated stopper. Although moulding may occur, the oil is now converted within a short period into a crust of fatty acid, and a burning candle, lowered into the flask is readily extinguished. This is another indication that neither too much oxygen nor its total absence is favorable for the formation of crusts of fatty acids. In fact such "atmospheric conditions" exist in and around coffins buried at greater depth.

In order to investigate to what extent other unsaturated oils would be subject to the microbial hardening process, the standard experiment was set up with 3% soybean oil, poppyseed oil, linseed oil, arachis oil, and triolein respectively, a flask with olive oil serving as a control. After 2 months' incubation the arachis oil flask showed the crust formation most strongly.

Finally the influence of temperature on the formation of fatty acids was studied. Flasks were incubated at 10°, 30°, and 37° C., and also at 25° C., the incubation temperature of the standard experiment. Crust formation appeared to proceed slowly at 10° C., though finally after half a year a distinct crust was noted. This is of major significance, as in our climate the soil temperature at a depth of 1 m and more is 9–10° C. When compared with 25° C. or room temperature, the process at 30° C. was somewhat retarded. At 37° C. crust formation was strongly inhibited; after a few months a wrinkled pellicle could be noted at the interface between oil and water, but after half a year the original volume of the oil which in the meantime had turned brown, seemed unchanged. The hardening process had clearly been brought to a standstill, probably because a group of the bacterial team had died off. In another experiment somewhat better results were obtained and a distinct crust formation could be noted. On the whole, however, 37° C. appears to be too high a temperature for the process.

B. Experiments in which the water phase is proteinaceous.

1. Experiments with garden soil as inoculum.

The formation of saturated fatty acids, adipocere, from human fat present in the dead bodies is induced by bacteria living under quite other conditions than those prevailing in the water phase of the standard experiment. Human tissue, particularly muscular tissue, contains about 20% protein and in order to meet the conditions prevailing in the decomposing bodies, experiments were undertaken with a water phase containing 20% peptone. To 2 L erlenmeyer flasks 1 L 20% peptone water was added, followed by 5% olive oil. Garden soil was used as inoculum and the flasks were incubated at 25° C.

Also here minimal quantities of garden soil may suffice to initiate the process.

Needless to state that now the microbial process was of quite another nature than in the former experiments. The bacteria now possessed a rich source of carbon, nitrogen and sulfur in the amply supplied peptone and did not have to depend on the laborious degradation of the olive oil in the upper layer. Accordingly, the lower layer was soon brought in a state of malodorous putrefaction. The gases which in earlier experiments had developed sparsily, now escaped abundantly through the perforated stoppers. In the initial experiments no crust formation could be noted after some months and, curiously enough, the oil appeared quite unattacked after half a year. A similar negative result was obtained after a flask had been inoculated with faeces. This result might be explained by the assumption that the lipolytic organisms in the soil sample (or faeces) had been destroyed during the putrefaction or that by toxic intermediary products or by the lack of a suitable source of nitrogen the oil had become resistant to microbial attack. Hence, the experiments with 20% peptone were repeated with another soil sample and in addition with peptone concentrations of 10 and 15%.

Now within even a shorter period of time than in the experiments with a mineral water layer, the olive oil appeared to be converted

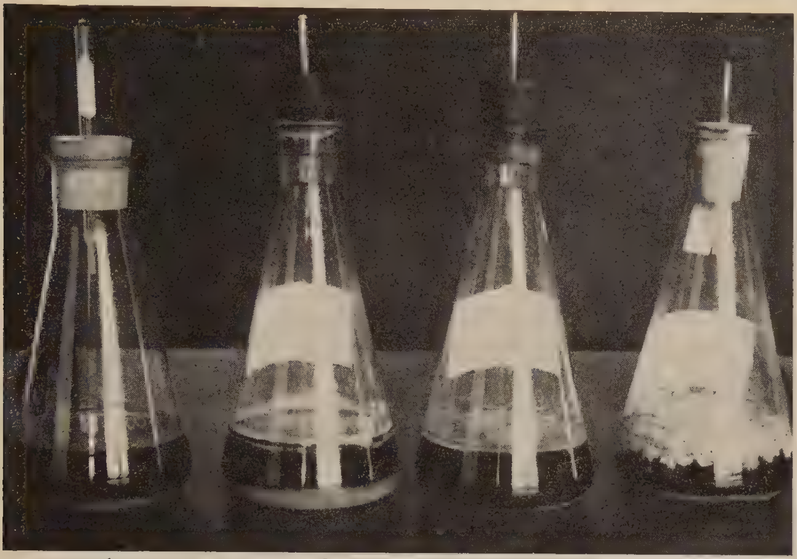

Fig. 2. Flasks with 20% peptone solution and 5% olive oil after incubation at 25° C. for 5 weeks. Flask (1) was not inoculated and served as a control; flask (2), inoculated with *Proteus vulgaris*, showed partial conversion; flask (3), inoculated with *Staphylococcus albus* showed somewhat less fatty acid formation and flask (4), inoculated with garden soil, showed total conversion of the olive oil.

into a thick crust of fatty acid, while moulding was completely absent (Fig. 2, flask 4).

With concentrations of 30% and higher crust formation failed to occur. Addition of 0.5% glucose to 20% peptone also inhibited crust formation. An extra supply of KNO_3 did not do so and addition of CO_2 appeared practically harmless.

Also here a burning candle was extinguished when brought into the flask. Hydrogen sulphide had developed abundantly. Thus, as in earlier experiments the atmosphere above the liquid surface was rich in carbon dioxide. Apparently the lipase-formers contained in the soil, in contradistinction to the former experiment, had had the opportunity to develop, and in this case had not to depend on a mineral source of nitrogen, like *Pseudomonas aeruginosa* discussed under A. Hydrogen liberated in putrefaction was probably responsible for the hardening of the unsaturated fatty acids from the olive oil. Thus the putrifying process supported the process of hardening.

Nevertheless, the above experiments have demonstrated the curious fact that the presence of peptone in the water phase does not necessarily involve the conversion of olive oil. The significance of this fact will be discussed later.

The above experiments were repeated with lower concentrations of peptone, *viz.* in erlenmeyer flasks with 1, 2, 3, 5 and 8% peptone water to which 5% olive oil had been added. Also here in 4 out of 5 flasks a strong crust formation could be noted after circa 6 weeks. This failed to occur, however, in 1% peptone, where, curiously enough, no carbon dioxide was demonstrable, an exception as peculiar as in the earlier experiment with 20% peptone. Apparently for the oxido-reduction of the fatty acids occurring in olive oil, a high concentration of peptone was not essential. Yet the crust formation with 5 to 8% peptone was stronger than with 2 and 3%.

When peptone solutions are allowed to putrify for a few days after inoculation with garden soil before the olive oil is added the chance of crust formation failing to occur is considerable.

Notwithstanding the irregularities which would occasionally occur, the *sub* B discussed method appeared to have important advantages over the mineral method. The crust formation was accelerated and occurred already after 10 days. Moreover bacteria with higher nutritional demands could achieve the splitting of the oil and the hardening of the fatty acids.

In order to investigate the influence of the depth of the oil layer on the formation of fatty acid crusts, 2.5, 5, 10, and 25% of olive oil was added to four 2 L flasks with 20% peptone water. After inoculation with garden soil these were incubated at 25° C. during 4 to 6 weeks. Just like in the cultures with a mineral water phase, crust formation occurred well with 2.5 and 5% olive oil, slightly with 10% and failed with 25% (Fig. 3). One flask with 20% peptone and 5% olive oil, incubated at 37° C. produced in the long run a crust, while a considerable part of the oil was left unconverted.

2. Experiments with pure cultures.

The isolation of 15 strains from a corpse converted into adipocere, offered a promising opportunity to obtain pure cultures capable of converting olive oil into crusts of fatty acid. These strains were representatives of the species *Proteus rettgeri*, *Proteus vulgaris*,

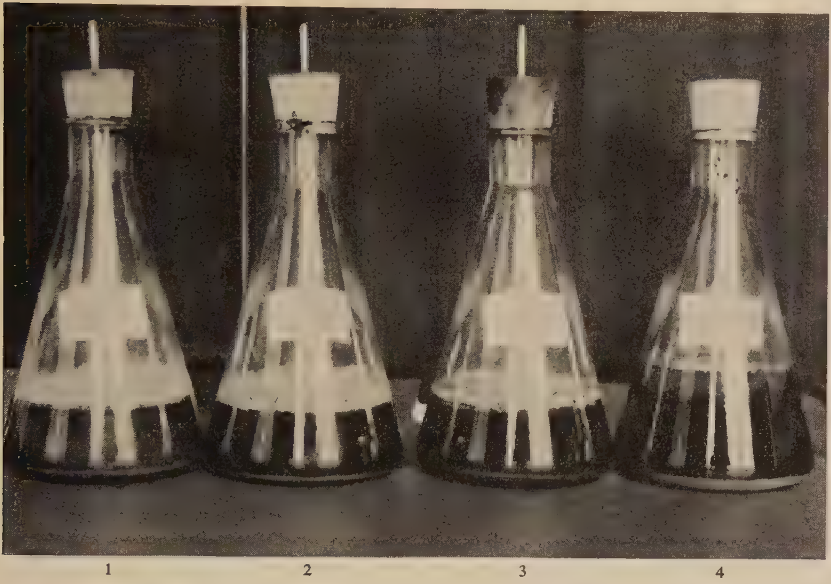

Fig. 3. Flasks with 20% peptone solution and different quantities of olive oil after incubation at 25° C. for 6 weeks. The flasks contained originally 2.5, 5, 10 and 25% of olive oil respectively (1, 2, 3, 4) and were inoculated with garden soil. In flasks (1) and (2) complete conversion into fatty acid crusts had occurred; flask (3) showed partial conversion and flask (4) none.

E. coli, *Staphylococcus albus*, *Staphylococcus aureus*, *Alcaligenes faecalis*, and enterococci and aerobic sporeformers of the *subtilis* group. They had been isolated from previously scorched parts of loin, thigh, breast, liver, brain and kidney. It is noteworthy that hardly any moulds were found in the organs and that in a considerable number of cases the material was found to be sterile. This becomes acceptable when taking into account that the human remains had been interred more than ten years earlier.

These 15 strains were inoculated into stoppered 0.5 l erlenmeyer flasks with 200 ml of 20% peptone water and 5% olive oil. After 4 to 5 weeks one strain of *Staphylococcus albus* (isolated from liver) and one of *Proteus vulgaris* (isolated from brain) appeared able to induce crust formation (Fig. 2, flasks 3 and 2). So did some months later a few other strains among which aerobic sporeformers. This was the first time that splitting of the olive oil and hardening of the fatty acid had been brought about by one single pure culture. In the remaining cases a milky turbidity had appeared in the oil layer, but crust formation was lacking. An analysis of the crust formed by two of the positive isolates is presented in Table 6.

TABLE 6.

Gaschromatographic analysis of olive oil and of the products formed from olive oil by pure cultures of bacteria isolated from interred corpses. Fatty acid quantities are expressed in % of weight.

Fatty acid	Olive oil	Product formed by	
		Staphylococcus albus 5A	*Proteus* 13B
Palmitic acid	10–12	32.5%	32.0%
Palmitoleic acid1)	1	1 to 1.5%	0.5 to 1 %
Stearic acid	2– 3.5	2.5%	2.5%
Oleic acid1)	70–83	60.5%	64.0%
Unidentified acid		1.5 to 2.0%	0.5 to 1.0%
Hydroxystearic acid	0	0.5 to 2. %	—
C_{20}	0	0.5 to 1.0%	0.2 to 0.5%

1) contains one double bond.

The analyses were performed by the Unilever Research Laboratory, Vlaardingen.

The experimental results led to an examination of present knowledge of lipase activity in *Staphylococcus*.

Eykman (1901) was the first to establish lipase in *Staphylococcus* and Wells and Corper (1912) studied it more closely. They noted that staphylococci possessed a greater lipase activity than several other bacterial species. Davies (1954) reported that the enzyme was widespread among staphylococci, but that no correlation existed between lipase and the origin of the strain. Lipase of staphylococci appeared fairly heat-stable, optimally active at pH 7.2 and generally was produced in an alkaline medium. Over 99.5% of the coagulase-positive strains of human origin appeared lipolytic, but among the animal strains only 75% possessed this property.

The above explains the fact that among the strains isolated from interred bodies, in the first place staphylococci had attracted attention by their crust formation in the oil.

In order to determine the optimal concentration of peptone, the most active strain of *Staphylococcus albus* (5A) was inoculated into stoppered erlenmeyer flasks with 5% olive oil and a water phase containing 2, 3, 5, 7, 10, 15 and 20% peptone respectively. After 6 weeks' incubation the oil with 2% peptone appeared merely granular, with 3% and 5% somewhat more so; with 7.5% the beginning formation of a white pellicle under the oil could be noted, with 10% this was somewhat thicker and with 15% and 20% a crust had developed in the oil. These experiments show that for optimal crust formation from olive oil by *S. albus* at least 15% of peptone is needed.

In view of the inhibitory action of glucose on crust formation, noted in soil enrichments, strain 5A was inoculated in stoppered flasks containing apart from peptone 1, 2, 3, and 5% of glucose respectively. Also here crust formation appeared to be inhibited by the presence of glucose. This was evidently due to the consumption of the nitrogen from the peptone in favour of this monose.

The latter observations motivated an investigation of a number of laboratory strains, first of all staphylococci, isolated from pus, throat swabs, faeces and the like. In order to economise on material 20 strains were inoculated in culture tubes which had been filled up to 2.5 cm from the bottom with 20% peptone water and then provided with 0.7 ml olive oil, forming a layer of 3 mm. After inoculation the tubes were closed with unperforated rubber stoppers and incubated in a vertical position at 25° C. After 6 weeks crusts appeared to have been formed under the oil in more than 90% of the cultures (Fig. 4).

These results showed that this capacity is practically a general property of staphylococci and not an accidental one of the strains isolated from interred bodies. Furthermore the experiment showed that the culture tube method served our purpose equally well as did cultivation in erlenmeyer flasks. While in garden soil enrichments, incubation at 25° C. had appeared favourable, this relatively low temperature was not needed for crust formation by pure cultures of staphyloccoci. At 37° C. the crusts were formed with equal ease and perhaps even more rapidly than at 25° C.

The fact that among the strains isolated from corpses which had been converted into adipocere, also a strain of *Proteus* had been encountered which could convert olive oil into fatty acid crusts, led to an investigation of a number of *Proteus mirabilis* strains from our culture collection. To our surprise after 4–6 weeks' incubation all of the 16 strains were found to have caused crust formation; 6 of them had achieved as strong a conversion as the staphylococci. Among the 12 strains of *Pseudomonas aeruginosa*, 6 possessed the property; the remaining ones were weak converters and 3 of these were practically inactive.

This demonstrated again that a process similar to formation of

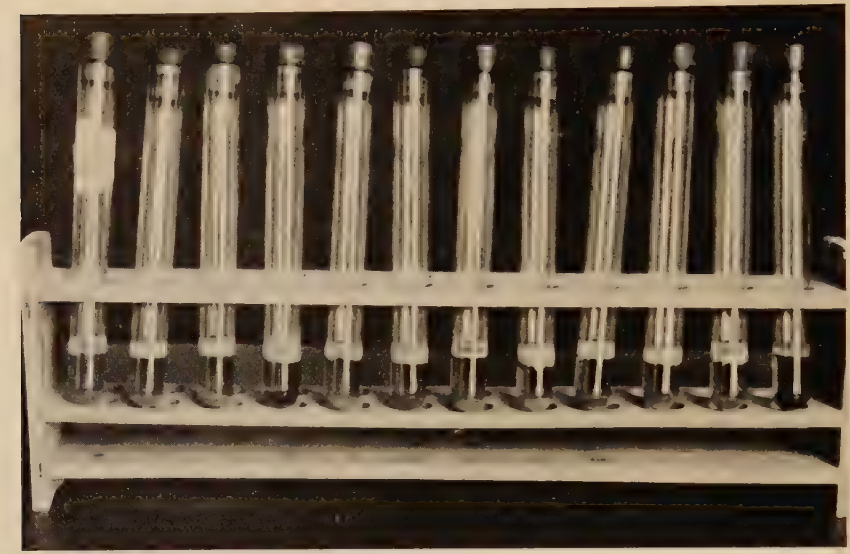

Fig. 4. Stoppered culture tubes with a layer of 25 mm of 20% peptone solution and of 3 mm of olive oil after incubation at 25° C. for 6 weeks. All tubes had been inoculated with a different strain of *Staphylococcus aureus* except the first one from the right, which served as a control. All inoculated tubes showed crust formation.

adipocere could be achieved *in vitro* by numerous bacteria which are present in dead bodies. It should be emphasized however, that such strains are not able to convert fats – comprising fats which will be considered in the next paragraph – in the presence of mineral nitrogen [$(NH_4)_2SO_4$] only.

After the experimental method had been greatly simplified by the use of the culture tubes, a number of other liquid fats, hence rich in unsaturated fatty acids, were studied. In the tubes the olive oil was replaced by triolein, soya-bean oil, arachis-oil, poppyseed-oil, and linseed-oil respectively and finally by human fat. Linseed-oil soon appeared less suitable because of its easy oxidation, leading to solidification. The other oils appeared sometimes less, sometimes more amenable than olive oil to conversion into crusts. It appeared that several strains of staphylococci, which practically all convert olive oil into thick crusts, could not achieve this with human fat. Apparently for this species olive oil is a more suitable substrate for the conversion, but for *Proteus* human fat appeared to be the better substrate.

DISCUSSION.

The processes which occur in flasks with a water phase of 20% peptone approach much more closely the processes in decomposing corpses than those in flasks with a mineral water phase. In the latter case only those bacteria from the garden soil will come to the fore, which are able to mineralise with ammonia as source of nitrogen the sole source of carbon present, *i.e.* the fat of olive oil. The processes in flasks with 20% peptone in the water phase are much more complicated.

After hydrolysis of the peptone, the resulting amino acids may be degraded in processes in which they serve as nitrogen as well as carbon and energy source; alternatively they may serve as nitrogen source in processes where the fats are dissimilated and function as carbon and energy source. Initially the former process in all probability greatly exceeds the latter: a strong putrefaction of the peptone occurs. Along with this, however, definite species of bacteria will come to the fore which are able to convert fats into saturated fatty acids, using the peptones and amino acids still present as an assimilation substrate.

Probably in this case the complete conversion is achieved by a single species of bacteria, in contradistinction to what occurs in the standard test, where collective action of a bacterial team appears to occur.

In some cases, however, the former process prevails to such an extent that the latter does not get any chance and the result will be that the oil remains unattacked. This exception will be the rule, when low concentrations of peptone, *i.e.* lower than 2% are used at the start. Experiments with pure cultures of *Staphylococcus aureus* confirmed that a decrease of the concentration of peptone is conducive to a decrease of the fat formation. A strongly proteinaceous nutrition thus appears favourable for crust formation. Curiously enough, however, crusts are formed as well when peptones and amino acids are completely absent as may be learned from the standard tests. The bacteria then have to depend on ammonia as source of nitrogen and the bacterial processes are of quite a different nature as is shown by the occurrence of *e.g.* sulphate reduction.

If we assume that initially the soil bacteria will not obtain entrance to the body, this being prevented by a solid coffin, it may be supposed that the processes mentioned will take place under the influence of the normal bacterial flora of the skin and the human mouth-anus duct. Pathologists accept as a fact that soon after death the intestinal bacteria invade the body. That these are able to convert fats into adipocere-like substances has been proved by experiments with pure cultures of *Proteus vulgaris* and even with *E. coli* (although here only to a small extent). The mouth and skin bacteria – particularly the staphylococci – are nutritionally more exacting than many bacteria in garden soil. They are generally unable to use ammonia as source of nitrogen like the latter and require amino acids, possibly together with vitamin-like substances, in order to successfully attack fats.

In the decomposing body both processes, putrefaction of protein and hardening of fatty acids can occur side by side. But under certain conditions only putrefaction will take place and the fat will remain unattacked. Some of the experiments *in vitro* have furnished proof for this possibility. Moreover by an increase of the concentration of CO_2 in and around the dead body and by a decrease in humidity also the putrefaction of the protein may be brought to a close. In fact it is probable that the porous adipocere by capillary action will attract a considerable amount of water and thereby decrease humidity. A drop of the relative humidity to as low as 15 or 10% generally brings all bacterial growth to a standstill.

As to the increase of the concentration of CO_2, it may be pointed out that gases in the abdominal cavity, obtained by punctioning the often strongly swollen body mainly consist of CO_2. In any decomposition of organic tissue CO_2 will develop. As in body cavities filled with CO_2 the inner organs are as a rule well conserved, be it strongly reduced in size, and decomposition phenomena are not met with on a large scale, even restricted decomposition apparently generates considerable amounts of CO_2. Laboratory experiments have demonstrated that organs kept in a atmosphere rich in CO_2 remain virtually unattacked during a long period of time.

Thus both processes, putrefaction of protein and hardening of fatty acid, can influence each other: strong putrefaction will check the formation of adipocere and the adipocere once formed will in its turn bring the putrefaction to a close. This mutual interaction may fluctuate in the course of time following changes in the environmental conditions. The remarkable phenomenon that organs in the cavities of breast and abdomen, although usually strongly reduced in size, are generally found to be well conserved, can be ascribed on one hand to the virtually anaerobic conditions in the coffin and the increase in CO_2 but on the other hand to desiccation.

The observations and hypotheses regarding the microbial processes are corroborated by pathological-anatomical data. Accordingly it has been noted several times that in parts of the mammae, within a layer of adipocere, islets of apparently unaltered yellow fatty tissue are present. Also in other parts of an adipocere corpse often unattacked fat and fatty substance, such as "butter" and oil can be found.

It need not be emphasized that in the presence of air microbial processes in experiments *in vitro* as well as in the dead body will take quite another course. Then along with bacteria, fungi will come to the fore, which will transform the proteins and fats completely to carbon dioxide and water. Formation of adipocere or preservation of fats is then excluded.

Summary.

The formation of adipocere is a process occurring under virtually anaerobic conditions in which human fat is converted into a complex of saturated fatty acids by a great variety of bacterial species occurring in and on the decomposing body.

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THE MYCETOME SYMBIONT OF *LASIODERMA SERRICORNE*

by

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(Received July 8, 1961).

INTRODUCTION.

Although ESCHERICH as early as 1900 first reported on the occurrence of a budding yeast-like symbiont in the digestive tract of *Stegobium,* there is no clarity on the characterization and classification of the symbionts of the *Anobiidae.* BUCHNER (1930) studied the symbionts of this family in some detail and isolated strains of a non-fermentative budding yeast-like organism which he temporarily named *Saccharomyces anobii.* PANT and FRAENKEL (1954), during studies on the role of the symbionts in the nutrition of *Lasioderma* and *Stegobium,* re-isolated strains which they considered were probably representatives of BUCHNER'S *Sacch. anobii.* From the description, however, it appeared that there was no justification for the assignment of the organism to the genus *Saccharomyces* (Meyen) Reess. In view of this and the fundamental role which the mycetome flora plays in the nutrition of the industrially significant anobiid beetles, a more detailed investigation of the mycetome symbiont of *Lasioderma serricorne* was undertaken.

MATERIALS AND METHODS.

The collection of the beetles and the subsequent dissection of the mycetomes were undertaken by Dr. D. L. MILNE of the Central Tobacco Research Station, Rustenburg, Transvaal.

Collection of beetles. Adult *Las. serricorne* beetles were collected from unbaited traps in co-operative tobacco sheds in Rustenburg.

Dissection of beetles. To avoid surface contamination by yeasts and moulds present on the exterior of the beetles, the insects were first submerged in a 0.5% solution of $HgCl_2$ for 5 minutes. After thorough and repeated washing with successive portions of sterile water, the beetles were transferred, ventral side up, on to sterile paraffin blocks under sterile water. The thorax was carefully withdrawn from the abdomen and the mycetome was dissected with suitable, sterilized scalpels and floated in sterile water.

Isolation. The mycetomes were transferred to, and smeared over the surface of plates prepared from Wickerham's stock culture agar (pH 3–4) and were incubated for 7 days at 28° C. Development of the symbiont was slow. Pure cultures of the organism were obtained by conventional plating techniques. Cultures of the symbiont could also be prepared by transferring the mycetomes to suitable liquid media.

Identification. Pure cultures of the symbiont were studied according to the standard methods of WICKERHAM (1951) and LODDER and KREGER-VAN RIJ (1952). Nuclear staining was done according to the method of GANESAN and SWAMINATHAN (1958).

RESULTS.

From eighteen dissected mycetomes virtually pure cultures of the mycetome symbiont were obtained.

Description of the symbiont.

Growth in malt extract: After 7 days at 25° C. cells of variable form are present (Fig. 1). Cells multiplying by bud-formation are round, oval to long-oval, or pyriform $(1.6–6.5) \times (2.6–7.8–10.5)\mu$, and occur singly, or in pairs, short chains or clusters. Cells multiplying by fission are round to cylindrical, occasionally elongated, $(2.5–6.5)\mu$ in diameter, and occur singly or in pairs or small groups. Growth is slow. Only a sediment is formed. After 1 month a ring and a sediment are formed.

Growth on malt agar: After 1 month at 17° C. the streak culture is butyrous, somewhat shiny, raised and yellowish-brown to ochraceous-salmon in colour. The margin is undulating, occasionally with some structure.

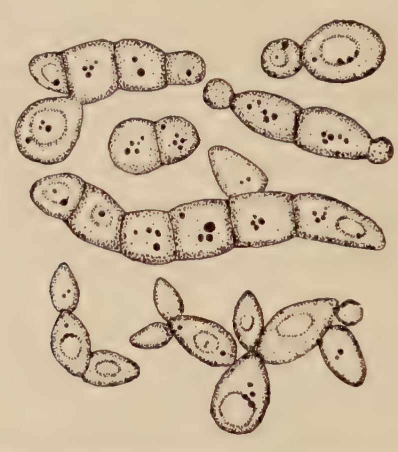

Fig. 1. Symbiont from *Las. serricorne* after 7 days in malt extract (1000 ×).

Nuclear condition: Young, mature cells are uninucleate.

Slide cultures: There is no formation of a characteristic pseudomycelium or filamentous hyphae.

Sporulation: Not observed[1]).

Fermentation: Absent.

Carbon assimilation: Glucose, galactose, L-sorbose, saccharose, maltose, trehalose, cellobiose, melibiose, raffinose, melizitose, D-arabinose, L-arabinose, D-xylose, D-ribose (slow), salicin, α-methyl-D-glucoside (weak), D-mannitol, D-sorbitol, *i*-inositol, dulcitol, adonitol, *i*-erythritol, glycerol, ethanol, potassium-D-gluconate (var.) and succinic acid (slow) are assimilated. Lactose, rhamnose, citric acid and lactic acid are not assimilated.

Assimilation of ethylamine: Positive.

Assimilation of nitrate and nitrite: Absent.

Growth in the absence of an extraneous vitamin source: Positive.

Growth at 37° C.: Absent.

Growth in osmotic pressure medium: Absent.

DISCUSSION.

As far as can be established, the isolated organism appears to correspond to the isolates of BUCHNER (1930) and PANT and FRAEN-

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[1]) Ascus formation was likewise not observed in any matings of 18 individual strains.

KEL (1954). It is, however, clear that the description of the symbiont as a yeast and its assignment to the genus *Saccharomyces* (Meyen) Reess must have been based solely on the grounds that the organism forms blastospores and produces butyrous, yeast-like colonies on solid media. The apparent absence of an ascogenous stage, the inability to evoke a gaseous fermentation, the characteristic cellular morphology and the pigmented growth on solid media not only debar the symbiont from the genus *Saccharomyces* but even exclude it from the yeast domain proper. The use of the epithet *Saccharomyces anobii* for the symbiont should therefore be discontinued.

The symbiont cannot be further classified at present with certainty. Supplementary data on the endobiontic phase of the symbiont, particularly as regards its nuclear condition and the possible existence of an alternation of generations within the mycetocytes, are required before any further classification can be attempted.

ROBERTS (1946a, b), however, amongst others, pointed out that the saprophytic haplophase of certain members of the Taphrinales is markedly yeast-like and could, when considered in the absence of the endobiontic, binucleate phase, be readily confused with the true anascosporogenous yeasts. In view of the rather strict host-specificity of the symbiont and the morphological and biochemical characteristics of the isolated strains, the possibility that it represents the haplophase of a member of the Taphrinales does not, therefore, seem to be excluded.

The fact that the isolated strains are capable of development in the absence of an extraneous vitamin source is in full agreement with the observations of PANT and FRAENKEL (1954) and BLEWETT and FRAENKEL (1943a, b) that the *Lasioderma* symbiont is a particularly efficient donor of the vitamins of the B-complex.

Summary.

Pure cultures of the mycetome symbiont of *Lasioderma serricorne* have been obtained and described. No detailed classification of the organism can be given at present.

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IMMUNOLOGICAL REACTIVITY OF TUBERCULOSIS PATIENTS

by

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(Received April 14, 1961).

With reference to a publication by Greenwood, Smellie, Barr and Cunliffe (1958) on the immunisability of sarcoidosis patients with tetanus toxoid, Wilkens and Tasman (1959) made a preliminary report on the immunological reactivity of tuberculosis patients to vaccination with tetanus phosphate toxoid (Tetanus-P.T.).

In that paper the antitoxin production in 17 tuberculosis patients was compared with that in a group of 11 "normal" controls. In both groups primary immunization was effected with two injections of 0.5 ml Tetanus-P.T. at an interval of about a month. The tuberculosis patients described were all from the Department of Pulmonary Diseases, University Hospital, Utrecht (Stads- en Academisch Ziekenhuis in Utrecht, abbreviated: SAZU).

This investigation showed that, on the whole, the tuberculosis patients reacted to this immunization by slower and less marked antitoxin production than did the controls. In view of the smallness of the material, however, this must be regarded as no more than a preliminary finding. Further investigation of the background of the phenomenon was impossible.

It seemed worth while to extend this investigation to cover a larger number of tuberculosis patients and controls. For this purpose we contacted the staff of the Tuberculosis Sanatorium "Zonnegloren" [1]). In this sanatorium, 37 patients were immunized against tetanus by two injections of 0.5 ml Tetanus-P.T. at a one-

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[1]) We are indebted to Dr. J. A. W. Berghauser Pont, medical director of the sanatorium "Zonnegloren", and Dr. J. Douma for their co-operation in this investigation.

TA

Controls showing a normal reaction (rapid

Number	Sex	Age in years	First titre at P.T.$_1$	Titre at P.T.$_2$	1st we
1	male	27	< 0.001	< 0.001	3.62
2	female	38	< 0.001	< 0.001	0.064
3	male	23	< 0.001	< 0.001	0.008
4	male	35	< 0.001	< 0.001	0.045
5	female	20	< 0.001	0.003	0.011
6	female	24	< 0.001	< 0.001	0.038
7	female	25	0.002	0.003	0.128
8	male	23	< 0.001	0.002	0.011
9	female	20	< 0.001	< 0.001	0.006
10	female	18	< 0.001	0.002	0.064
11	female	39	< 0.001	< 0.001	0.016
12	male	22	< 0.001	< 0.001	0.023
13	female	22	< 0.001	< 0.001	0.004
14	male	24	< 0.001	0.002	0.032
15	female	22	0.001	0.002	0.064
16	male	24	< 0.001	0.004	0.004
17	male	30	< 0.001	< 0.001	0.023
18	male	47	< 0.001	0.001	0.011
19	female	34	< 0.001	< 0.001	0.045
20	male	39	< 0.001	< 0.001	0.011
21	female	36	< 0.001	0.001	0.023
22	male	36	< 0.001	0.001	0.045
23	female	54	< 0.001	0.001	0.016
		Geometric mean:	0.005	0.001	0.026

TA

Controls showing a delayed reaction to primary immuniza

Number	Sex	Age in years	First titre at P.T.$_1$	Titre at P.T.$_2$	1st wee
24	male	26	0.001	< 0.001	< 0.00
25	male	28	< 0.001	< 0.001	< 0.00
26	female	27	< 0.001	< 0.001	< 0.00
27	female	24	0.001	< 0.001	0.00
28	male	23	0.001	< 0.001	< 0.00
29	male	48	< 0.001	< 0.001	0.00
		Geometric mean:	0.0007	0.0005	0.00

a satisfactory height) to primary immunization.

Titration results AU/ml					
nd week	3rd week	4th week	5th week	6th week	7th week
2.56	—	2.30	2.30	—	—
0.256	0.600	1.10	0.60	0.23	0.16
0.011	0.020	0.016	—	—	—
0.045	0.040	—	0.023	—	—
0.110	0.080	—	0.160	—	—
0.160	0.110	—	0.040	—	—
0.040	0.040	—	0.110	—	—
0.045	0.045	0.040	—	—	—
0.362	1.28	2.30	—	—	—
0.128	0.570	0.400	—	—	—
0.032	0.080	0.110	—	—	—
0.256	2.30	1.10	—	—	—
0.032	0.016	—	—	—	—
0.064	0.450	0.450	—	—	—
0.064	0.160	0.110	—	—	—
0.256	1.10	0.400	—	—	—
0.045	0.570	0.300	0.110	—	—
0.064	0.045	0.110	0.110	0.110	0.110
0.064	0.045	0.160	0.110	0.110	0.110
0.011	0.011	0.023	0.011	0.011	0.003
0.256	1.10	3.20	1.60	1.60	1.10
0.090	0.230	0.570	0.400	0.080	0.011
0.128	0.230	—	—	—	0.110
0.089	0.152	0.267	0.156	0.126	0.069

to a satisfactory height (with the exception of one subject).

Titration results AU/ml					
nd week	3rd week	4th week	5th week	6th week	7th week
—	—	1.81	—	—	1.60
0.064	0.320	0.032	0.032	—	—
—	0.181	—	0.900	0.300	—
0.045	0.040	0.011	—	—	—
0.064	—	0.160	—	—	—
0.016	0.006	0.008	0.004	0.002	0.001
0.042	0.062	0.061	0.033	0.016	

TA

Tuberculosis patients showing a normal reac

Number	Sex	Age in years	Clinic	First titre at P.T.$_1$	Titre at P.T.$_2$	1st w
1	male	52	Zonnegloren	< 0.001	< 0.001	0.00
2	male	23	Zonnegloren	< 0.001	0.008	0.25
3	male	33	Zonnegloren	0.004	0.002	0.04
4	male	20	Zonnegloren	< 0.001	0.002	0.02
5	male	19	Zonnegloren	< 0.001	< 0.001	0.12
6	male	42	Zonnegloren	< 0.001	< 0.001	0.04
7	male	45	Zonnegloren	< 0.001	< 0.001	0.01
8	male	22	Zonnegloren	< 0.001	0.001	0.00
9	male	21	Zonnegloren	< 0.001	< 0.001	0.02
10	male	27	Zonnegloren	< 0.001	0.001	0.01
11	female	50	Zonnegloren	0.001	0.002	0.00
12	female	36	Zonnegloren	0.001	< 0.001	0.01
13	female	29	Zonnegloren	< 0.001	< 0.001	0.00
14	female	39	Zonnegloren	< 0.001	< 0.001	0.01
15	female	58	Zonnegloren	< 0.001	< 0.001	0.00
16	female	32	Zonnegloren	< 0.001	0.002	0.02
17	female	57	Zonnegloren	< 0.001	< 0.001	0.00
18	female	41	Zonnegloren	< 0.001	< 0.001	0.02
19	female	53	Zonnegloren	< 0.001	< 0.001	0.01
20	female	21	Zonnegloren	< 0.001	0.001	0.02
21	female	43	Zonnegloren	< 0.001	< 0.001	0.00
22	female	28	Zonnegloren	< 0.001	< 0.001	0.02
23	female	31	Zonnegloren	< 0.001	0.001	0.01
24	female	24	S.A.Z.U.	< 0.001	—	0.01
25	male	37	S.A.Z.U.	0.002	< 0.001	0.02
26	male	50	S.A.Z.U.	0.001	< 0.001	0.00
			Geometric mean:	0.0006	0.0008	0.01

month interval. Blood samples were obtained from these patients before, during and after this primary immunization, and the development and course of immunity were followed by means of serum antitoxin determinations. For the technique of these antitoxin determinations we refer to the publication of Wilkens and Tasman (1959).

With the above mentioned 17 cases from the SAZU, we had a group of 54 patients with manifest signs and symptoms of tuberculosis at the time of the investigation or in the recent past.

For comparison we used a control group of 29 normal subjects –

d and to a satisfactory height) to primary immunization.

ration results AU/ml							
week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week
128	—	0.160	0.160	0.080	0.064	0.032	0.032
30	—	0.450	0.320	0.230	0.181	0.810	0.045
032	—	0.045	0.090	0.023	0.023	0.032	0.023
064	—	0.090	0.090	0.032	0.045	0.045	0.045
450	—	0.450	0.320	0.230	0.090	0.128	0.023
045	—	0.032	0.023	0.011	0.016	0.016	0.016
023	—	—	—	—	—	—	—
032	0.032	—	0.032	0.032	0.023	0.023	0.032
032	0.016	—	0.016	0.016	0.016	0.023	0.023
016	0.032	—	0.011	0.011	0.006	0.011	0.008
045	0.045	0.016	—	0.016	0.023	0.023	0.023
032	0.023	0.032	—	0.011	0.016	0.011	0.011
016	0.016	0.016	—	0.011	0.016	0.008	0.011
064	0.362	0.160	—	0.045	0.045	0.045	0.045
090	0.300	0.110	—	0.030	0.023	0.016	0.023
181	1.100	1.100	—	0.320	0.320	0.160	0.128
045	0.045	0.230	—	0.060	0.064	0.023	0.023
230	0.080	0.060	—	0.011	0.016	0.008	0.004
090	0.230	0.060	—	0.032	0.016	0.016	0.008
230	0.230	0.230	—	0.160	0.230	0.230	0.320
064	0.230	0.110	—	0.045	0.023	0.023	0.023
450	0.160	0.160	—	0.045	0.045	0.045	0.045
900	0.800	0.570	—	0.160	0.110	0.090	0.090
—	—	0.023	—	—	—	—	—
016	0.090	0.090	0.128	0.032	0.016	—	—
032	0.032	0.032	0.032	0.006	—	—	—
080	0.096	0.099	0.064	0.036	0.036	0.035	0.027

all known to be free of active tuberculosis at the time of the investigation, to be working normally, not to use tuberculostatics and to have a history negative for treatment for any tuberculous affection.

The results of this immunization are summarized in Tables 1–5, and presented also in graphs in Figures 1 and 2. For the controls and for the tuberculosis patients we used an (arbitrary) classification of "normal reaction", "delayed reaction" and "no or hardly any reaction". The term "delayed reaction" as used here refers to the cases in which no unmistakable rise in titre occurred within one week of the second vaccine injection. Titre values < 0.001 AU/ml

TAB

Tuberculosis patients showing a delay

Number	Sex	Age in years	Clinic	First titre at P.T.$_1$	Titre at P.T.$_2$	1st we
27	male	42	Zonnegloren	< 0.001	< 0.001	0.0
28	male	59	Zonnegloren	0.001	0.001	0.0
29	male	25	Zonnegloren	< 0.001	< 0.001	0.0
30	male	44	Zonnegloren	< 0.001	< 0.001	< 0.0
31	male	54	Zonnegloren	< 0.001	< 0.001	0.0
32	female	37	Zonnegloren	< 0.001	< 0.001	0.0
33	female	62	Zonnegloren	< 0.001	< 0.001	< 0.0
34	female	39	Zonnegloren	< 0.001	< 0.001	0.0
35	female	29	Zonnegloren	< 0.001	0.001	0.0
36	female	41	Zonnegloren	< 0.001	< 0.001	0.0
37	male	34	S.A.Z.U.	< 0.001	< 0.001	< 0.0
38	male	42	S.A.Z.U.	< 0.001	0.001	< 0.0
39	male	49	S.A.Z.U.	< 0.001	< 0.001	< 0.0
40	male	22	S.A.Z.U.	< 0.001	< 0.001	< 0.0
			Geometric mean:	0.0005	0.0006	0.0

TAB

Tuberculosis patients showing lit

Number	Sex	Age in years	Clinic	First titre at P.T.$_1$	Titre at P.T.$_2$
41	male	51	Zonnegloren	< 0.001	< 0.001
42	male	46	Zonnegloren	< 0.001	< 0.001
43	female	39	Zonnegloren	< 0.001	< 0.001
44	female	30	S.A.Z.U.	< 0.001	< 0.001
45	female	27	S.A.Z.U.	< 0.001	< 0.001
46	male	42	S.A.Z.U.	< 0.001	0.001
47	male	27	S.A.Z.U.	0.001	< 0.001
48	male	35	S.A.Z.U.	< 0.001	< 0.001
49	male	50	S.A.Z.U.	< 0.001	< 0.001
50	male	45	S.A.Z.U.	< 0.001	0.001
51	male	15	S.A.Z.U.	< 0.001	< 0.001
52	female	46	Zonnegloren	< 0.001	< 0.001
53	male	50	S.A.Z.U.	< 0.001	< 0.001
54	female	33	S.A.Z.U.	< 0.001	< 0.001
			Geometric mean:	0.0005	0.000

slow reaction to primary immunization.

tration results AU/ml							
week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week
.006	—	0.006	0.006	0.006	0.004	0.008	0.008
.016	—	0.006	0.006	0.003	0.004	0.006	0.003
.181	0.320	—	0.230	0.320	0.230	0.256	0.230
.023	0.004	—	0.004	0.004	0.004	0.003	0.002
.011	0.032	—	0.003	0.006	0.006	0.006	0.006
.032	0.023	0.016	—	0.006	0.008	0.004	0.006
.045	0.045	0.032	—	0.011	0.016	0.011	0.006
.256	0.640	0.640	—	0.160	0.230	0.110	0,008
.045	0.110	0.110	—	0.032	0.045	0.064	0.064
.016	0.016	0.008	—	0.006	0.008	0.006	0.040
—	—	0.032	—	—	—	—	—
.003	0.008	0.016	0.045	—	—	—	—
.090	0.367	0.640	0.320	0.320	—	—	—
.128	0.090	0.080	—	0.011	—	—	—
.032	0.055	0.039	0.020	0.018	0.016	0.014	0.012

no reaction to primary immunization.

tration results AU/ml					
st week	2nd week	3rd week	4th week	5th week	6th week
0.001	0.004	0.016	—	0.004	0.004
< 0.001	0.004	0.008	—	0.004	—
0.001	0.002	0.004	0.004	—	0.002
0.001	—	—	—	—	—
—	—	—	—	0.008	—
0.001	< 0.001	0.002	0.002	< 0.001	0.001
< 0.001	0.001	0.004	0.002	0.001	0.006
< 0.001	—	0.008	< 0.001	< 0.001	—
< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	—
< 0.001	0.001	0.003	0.003	0.003	0.003
< 0.001	0.001	0.001	< 0.001	< 0.001	< 0.001
0.001	0.004	0.008	—	—	—
< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
0.001	0.003	< 0.001	< 0.001	—	—
0.0007	0.0015	0.0026	0.0010	0.0010	0.0018

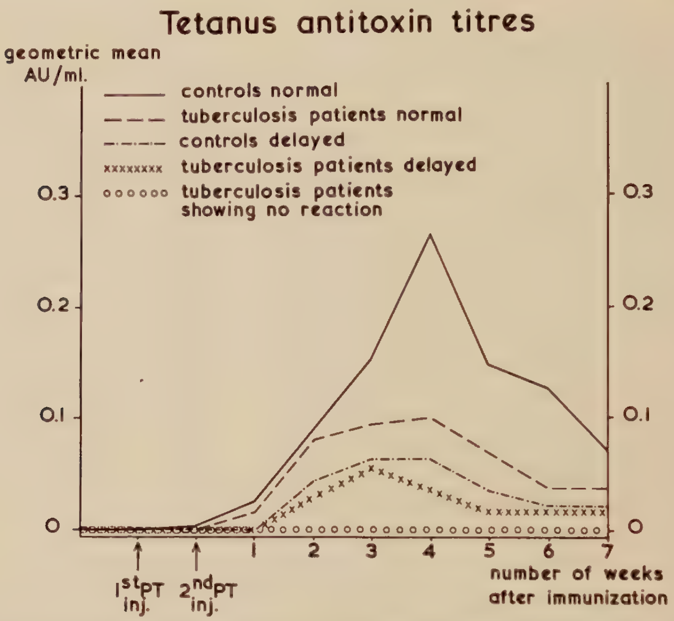

Fig. 1. Comparison of reaction of tuberculosis patients with that of controls.

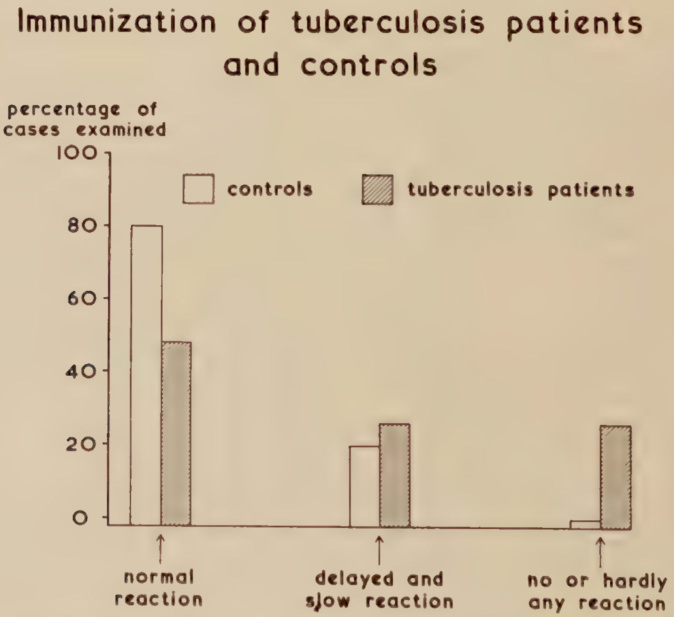

Fig. 2. Comparison of reaction of tuberculosis patients with that of controls.

were estimated at 0.0005 AU/ml for calculation of the geometric mean.

The above tables and figures warrant the following conclusion. Of the 29 controls, 23 (80%) showed a good reaction to primary immunization, while 6 (20%) showed a delayed reaction. Of the 54 tuberculosis patients, 26 (48%) showed a good reaction; 14 (26%) showed a delayed reaction, and 14 (26%) little or no reaction. The tuberculosis patients as a group showed an unmistakably less good reaction to immunization than did the normal controls. This finding confirms the tentative conclusions formed by Wilkens and Tasman (1959).

It will be demonstrated that this chiefly concerns an unfavourable effect with regard to primary immunization. In 27 of the patients included in this investigation we studied the effect of a booster injection (0.5 ml Tetanus-P.T.). The results are presented in Table 6.

This shows that all patients except No. 49 and No. 50 presented a normal reaction to this booster injection. This is true in particular of patients 27, 28, 29, 31, 32, 33, 41, 43, 46 and 54, who showed no or only delayed antitoxin production after primary immunization.

The results so far presented corroborate those of Greenwood, Smellie, Barr and Cunliffe (1958).

On submitting the facts presented to a more detailed scrutiny, we find that the relatively small group of SAZU patients on the whole showed a considerably less favourable reaction to the antigen injections than did the "Zonnegloren" patients (Table 7).

In addition it should be pointed out once again that, on the whole, the "normally" reacting tuberculosis patients clearly lagged behind the normally reacting controls. We have attempted to establish the extent to which this difference between SAZU and 'Zonnegloren" patients might be explained on the basis of a difference between the two groups as to the nature or extent of the tuberculous process, age, sex and treatment received.

The majority of patients in both groups were suffering from pulmonary tuberculosis (15 of 17 SAZU and 30 of 37 "Zonnegloren" patients). In 17 cases (9 SAZU and 8 "Zonnegloren"), there was cavitary tuberculosis at the time of the investigation or in the recent past. The severity of the disease (relatively more cases of cavitary tuberculosis among the SAZU than among the "Zonnegloren" patients) might seem to explain the difference in antitoxin formation, but the figures available are too small to have any real

TABLE 6.

Reaction to booster injection in tuberculosis patients.

No patient	Titre 3-4 weeks after 2nd P.T. injection	Months between 2nd P.T. and booster injection	Titre at moment of booster injection	Titre 7 days after booster injection
1	0.160	6.5	0.003	1.10
4	0.090	6.5	0.320	1.10
6	0.032	6.5	0.032	0.56
9	0.032	6.5	0.128	4.50
10	0.032	6.5	0.032	0.15
11	0.016	6.5	0.032	2.30
12	0.032	6.5	0.045	0.56
13	0.016	6.5	0.900	1.10
14	0.160	6.5	0.450	9.00
15	0.110	6.5	0.032	0.56
18	0.060	6.5	0.016	0.30
19	0.060	6.5	0.011	0.56
21	0.110	6.5	0.064	1.10
23	0.570	6.5	0.128	0.15
25	0.090	9	0.032	0.95
27	0.006	6.5	0.016	4.50
28	0.006	6.5	0.016	0.56
29	0.320	6.5	0.032	9.00
31	0.032	6.5	0.004	0.56
32	0.016	6.5	0.023	2.30
33	0.032	6.5	0.003	0.30
41	0.016	6.5	0.090	0.30
43	0.004	6.5	0.008	0.10
46	0.002	8	0.001	0.13
49	< 0.001	8	< 0.001	< 0.001
50	0.003	10	0.002	0.004
54	< 0.001	11	< 0.001	0.13

significance. The presence or absence of positive sputum findings, the extent of the lesions or the duration of the tuberculous history had no demonstrable influence in this respect. A striking feature is (although the number is small) that 6 patients who had undergone the stress of a thoracotomy shortly before examination (patients 5, 20, 34, 36, 37, 38) included 4 with abnormal reactions, and that 2 patients who were severely ill (Nos. 33 and No. 51: primary tuberculosis with a large obstructive infiltrate and bone tuberculosis with miliary dissemination) both showed abnormal titre curves (see Tables 4 and 5).

TABLE 7.

Difference in reaction between SAZU patients and "Zonnegloren" patients.

Clinic	Total	Abnormal reaction	Normal reaction
S.A.Z.U.	17	14 (82%)	3 (18%)
"Zonnegloren"	37	14 (38%)	23 (62%)
Controls	29	6 (20%)	23 (80%)

TABLE 8.

Distribution of patients according to sex and immunological reaction.

Clinic	Men total	Abnormal reaction	Women total	Abnormal reaction
S.A.Z.U.	13	11 (85%)	4	3 (75%)
"Zonnegloren"	18	8 (44%)	19	6 (32%)
Controls	15	4 (27%)	14	2 (14%)

TABLE 9.

Distribution of patients according to age and immunological reaction.

Clinic	15–40 years	Abnormal reaction	40 years and over	Abnormal reaction
S.A.Z.U.	10	7 (70%)	7	6 (86%)
"Zonnegloren"	19	5 (26%)	18	9 (50%)
Controls	26	0 (—)	3	1 (30%)

The sex distribution was as indicated in Table 8.

The overall percentage of abnormal reactions showed a slight male predominance. Although the SAZU group consisted chiefly of males, the percentual differences in reaction are small, and the absolute numbers likewise. It is not yet possible to determine the importance of sex as a factor in this phenomenon.

The age distribution is presented in Table 9.

The marked difference in reaction between the SAZU and the "Zonnegloren" patients cannot be explained by the fact that the older patients showed a slightly less favourable immunological reaction than the younger ones (particularly among the "Zonnegloren" group). In fact 41% of the SAZU group were over 40, while the corresponding percentage in the "Zonnegloren" group was 49.

Distribution according to medication (PAS and isoniazid) is shown in Table 10.

TABLE 10.

Distribution of patients according to medication and immunological reaction.

Clinic	P.A.S. during experiment	Abnormal reaction	I.N.H. during experiment	Abnormal reaction
S.A.Z.U.	12¹)	9 (75%)	14²)	11 (79%)
"Zonnegloren"	34³)	14 (41%)	35³)	14 (40%)

¹) Ten of these 12 patients received the P.A.S. intravenously, 7 of these 10 patients showed an abnormal reaction.

²) Eleven of these 14 patients received the I.N.H. intravenously, 8 of these 11 patients showed an abnormal reaction.

³) The "Zonnegloren"-patients received the P.A.S. and I.N.H. orally.

At the time of the investigation, nearly all patients examined were receiving PAS and isoniazid. The few who were not receiving such medication had been treated with these agents in the recent past.

Other tuberculostatics used, *e.g.* streptomycin, cycloserine and pyrazinamide, can be disregarded because only few patients were being treated with these agents at the time of the investigation (1 SAZU and 5 "Zonnegloren" cases received streptomycin; 3 SAZU and 1 "Zonnegloren" cycloserine; 3 SAZU patients pyrazinamide).

It seems an obvious possibility that rather than the tuberculous disease as such, the PAS and isoniazid medication might have been responsible for the abnormal titre course following immunization with Tetanus-P.T. There might be a correlation between the larger percentage of abnormal reactions in the SAZU group than in the "Zonnegloren" group and the mode of administration of PAS and isoniazid. The SAZU patients were as a rule treated by intravenous injection, whereas the "Zonnegloren" patients received oral medication. It is conceivable that intravenous PAS administration (15 g daily by drip infusion for 3 hours) might give higher blood concentrations than oral administration (12 g daily distributed over the day).

Summarizing the facts so far presented it can be stated that:

In the course of the disease there occurs an inhibition of the ability to form antibodies in the "normal" manner on primary immunization (in our case with Tetanus-P.T.), possibly as a result of a (transient) injury of the RES. Whether this effect is specific, *i.e.* dependent on the tubercle bacilli involved or their products, cannot be definitely stated at this time. As mentioned at the beginning of this paper, GREENWOOD *et al.* (1958) observed the same phenomenon in patients suffering from sarcoidosis[1]).

It is also conceivable, however, that this abnormal immunological reactivity is due not so much to the pathological process as such but chiefly to the PAS and/or isoniazed medication employed.

In this connection there are three possibilities *viz.*:

a. The presence of PAS and isoniazid in the serum exerts and unfavourable influence on antitoxin determinations;
b. PAS and isoniazid have an unfavourable effect on the antigen (tetanus toxoid), as a result of which the latter is less efficacious;
c. PAS and isoniazid have an injurious effect on the RES, so that the desired immunity develops less well.

a. Does the presence of PAS and/or isoniazid in the serum influence the antitoxin determination?

One non-immunized rabbit was intravenously injected with 200 mg PAS sodium in 2 ml salt solution, and another with 1 mg isoniazid in 1 ml salt solution. The PAS or isoniazid concentration in blood obtained by venepuncture was determined after 15 minutes, 30 minutes, 1 hour and 2 hours[2]). Clearly demonstrable quantities (0.9 mg PAS/100 ml and 0.17 mg isoniazid/100 ml) were still demonstrable 1 hour after administration.

Subsequently, 4 rabbits were immunized by 2 injections of 0.5 ml Tetanus-P.T. at a one-month interval, and four months after the second antigen injection the antitoxin titres in the blood were determined. Then the rabbits received the following intravenous injections:

Rabbit 1: 200 mg PAS sodium in 2 ml salt solution;
Rabbit 2: 1 mg isoniazid in 1 ml salt solution;
Rabbit 3: 200 mg PAS sodium and 1 mg isoniazid in 2 ml salt solution;
Rabbit 4: 2 ml salt solution.

Another venepuncture was made 15 minutes after the injection of PAS or isoniazid and the drug concentrations and antitoxin titres were determined.

The results of these experiments are given in Table 11.

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[1]) BERGQUIST and PACKALÉN (1958) observed the reverse phenomenon in tuberculous guinea-pigs. These animals, after spontaneous infection with staphylococci and immunization with α-staphylolysin and streptolysin-O, showed higher antibody titres than non-tuberculous guinea-pigs.

[2]) The PAS and isoniazid determinations mentioned were carried out by Dr. A. H. HOLTZ, Chemical Department, National Institute of Public Health.

TABLE 11.

Antitoxin titres in rabbits before and immediately after administration of PAS or isoniazid.

Number rabbit	Titre before immunisation AU/ml	Titre 4 months after immunisation, before injection of P.A.S. and I.N.H.	mg P.A.S. and/or I.N.H. injected	15 min. after P.A.S. and/or I.N.H. injection	
				Titre AU/ml	mg % P.A.S. and/or I.N.H. in serum
1	< 0.001	2.30	200 P.A.S.	2.30	3.5
2	< 0.001	0.10	1 I.N.H.	0.10	0.19
3	< 0.001	0.60	200 P.A.S. +1 I.N.H.	0.60	3.2 P.A.S. + 0.2 I.N.H.
4	< 0.001	1.60	——	1.60	

These results show that the presence of PAS or isoniazid does not influence the antitoxin determination.

b. Do PAS and isoniazid exert an unfavourable effect on the antigen (tetanus toxoid)?

16 ml Tetanus-P.T. was divided into 4 equal parts and treated as follows:

a. 4 ml Tetanus-P.T. was mixed with 200 mg PAS sodium in 2 ml salt solution, and with 2 ml salt solution;
b. 4 ml Tetanus-P.T. was mixed with 2 mg isoniazid in 2 ml salt solution, and 2 ml salt solution.
c. 4 ml Tetanus-P.T. was mixed with 200 mg PAS sodium and 2 mg isoniazid in 4 ml salt solution.
d. 4 ml Tetanus-P.T. was mixed with 4 ml salt solution.

The mixtures obtained were stored for 24 hours at room temperature, centrifuged, washed twice in salt solution and then made up with salt solution to the original 4 ml volume.

The Tetanus-P.T. samples thus treated were tested for immunizing potency in a mouse protection test by the method of Cohen, van Ramshorst and Tasman (1959). For comparison we used the Dutch Standard P.T., the number of protective units of which has been set at 100/ml.

Results of this experiment are presented in Table 12.

The differences between results with the treated Tetanus-P.T. and those with the Standard-P.T. are within the limits of error of the determination. This warrants the conclusion that PAS, isoniazid

TABLE 12.

Determination of immunizing potency of tetanus-P.T. treated with PAS, isoniazid, PAS and isoniazid, and salt solution.

Vaccine	Protective Units/ml
Dutch reference P.T.	100
Tetanus P.T., treated with P.A.S.	87
Tetanus P.T., treated with I.N.H.	67
Tetanus P.T., treated with P.A.S. + I.N.H.	81
Tetanus P.T., treated with saline	79

or a combination of the two does not unfavourably affect the antigenic power of Tetanus-P.T. treated with these agents.

c. **Do PAS and isoniazid exert an injurious effect on the RES, as a result of which the desired immunity develops less well?**

To answer the question whether protracted treatment with PAS and isoniazid before and during immunization with Tetanus-P.T. unfavourably influences the immunization response, the following tests were carried out.

Sixty-seven guinea-pigs were divided into 4 groups and treated as follows:

Group 1: 21 guinea-pigs received daily subcutaneous injections of 50 mg PAS sodium in 1 ml salt solution for 10 weeks. One month after the first PAS injection they received 0.5 ml Tetanus-P.T. intramuscularly. Three weeks later blood was taken by heart puncture. Three days later they received the second intramuscular P.T. injection, and a further three weeks later they were bled.

Group 2: 16 guinea-pigs were given daily subcutaneous injections of 6 mg isoniazid in 1 ml salt solution for 10 weeks. Subsequently they were treated as described for group 1.

Group 3: 11 guinea-pigs were given daily subcutaneous injections of a mixture of 50 mg PAS sodium and 6 mg isoniazid in 2 ml salt solution during 10 weeks. Further treatment as described.

Group 4: 19 guinea-pigs were given daily subcutaneous injections of 1 ml salt solution for 10 weeks. Further treatment as described.

Antitoxin titres were determined by the haemagglutination technique described by TASMAN, VAN RAMSHORST and SMITH (1960) for titres 0.01 AU/ml, and by the titration technique of WILKENS and TASMAN (1959) for titre values < 0.01 AU/ml. Titres < 0.001 AU/ml were estimated at 0.0005 AU/ml.

The antitoxin titres found 3 weeks after the first P.T. injection are presented in Table 13.

TABLE 13.

Antitoxin titres in guinea-pigs treated before, during and after tetanus immunization with PAS, isoniazid, PAS and isoniazid or salt solution. Antitoxic titres 3 weeks after 1st P.T. injection:

Group 1 P.A.S. AU/ml	Group 2 I.N.H. AU/ml	Group 3 P.A.S. + I.N.H. AU/ml	Group 4 saline AU/ml
0.079	0.001	0.126	0.001
0.032	1.00	0.012	2.00
0.001	0.316	0.001	0.251
0.260	2.00	2.51	0.251
0.001	3.16	0.001	0.398
0.001	0.316	0.050	2.00
0.031	0.631	0.398	1.58
0.001	0.002	0.251	0.002
0.100	0.032	0.398	2.00
0.040	0.631	0.316	0.200
0.100	0.251	0.001	1.00
0.0005	0.251		0.158
0.001	1.26		0.501
0.001	7.94		0.501
0.050	0.0005		3.16
0.050	0.032		1.00
0.0005			1.00
0.631			1.26
0.126			1.00
0.0005			
0.631			
0.012	0.183	0.121	0.393

TABLE 14.

Analysis of variance of the titration results presented in Table 13.

Effect due to	Sum of squares	Degrees of freedom	Mean sum of squares	F	P
P.A.S.	24.11	1	24.11	19.0	< 0.0001
I.N.H.	3.70	1	3.70	2.9	0.1
Interaction P.A.S. × I.N.H.	3.95	1	3.95	3.1	0.009
Rest	79.86	63	1.27	—	—

The results were statistically analysed1). As usual, the calculation was applied to the logarithms of the titre values. Analysis of variance

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1) Statistical analysis by Mr. G. J. Leppink of the Department of T.N.O., The Hague.

according to SNEDECOR (1946) yielded the features presented in Table 14.

The figures given in Table 13, and the analysis of variance applied to them, warrant the following conclusions. PAS treatment of guinea-pigs before and during immunization with one injection of 0.5 ml Tetanus-P.T. has an unmistakable inhibitory influence on the production of tetanus antitoxin ($P < 0.0001$).

The analysis of variance failed to demonstrate such an influence of isoniazid, either as "main effect" or as component of interaction.

Three weeks after the second P.T. injection the animals were bled and antitoxin titres in their serum were determined. In all groups the animals were found to have reached a "normal" antitoxin titre, as shown in Table 15.

TABLE 15.

Antitoxin titres in guinea-pigs treated with PAS, isoniazid, PAS and isoniazid, or salt solution, three weeks after the second tetanus-P.T. injection.

Group	Treated with	Geometric mean 3 weeks after 2nd Tetanus P.T.-injection, AU/ml
1	P.A.S.	12.6
2	I.N.H.	18.0
3	P.A.S. + I.N.H.	6.7
4	Saline	17.1

The results obtained in the above groups lead to the following conclusions:

1. The presence of PAS or isoniazid in the serum does not interfere with antitoxin determination;
2. PAS and isoniazid have no injurious effect on the vaccine used;
3. Protracted PAS medication of guinea-pigs before and during immunization with one injection of Tetanus-P.T. has an unfavourable effect on the production of antitoxin.

Considering the previously presented results of immunization of tuberculosis patients and normal controls in the light of these experimental data, the following (tentative) conclusions can be drawn.

It is possible that the less favourable results of primary immunization of tuberculosis patients may be due – apart from obscure causes in the tuberculous process as such – to the tuberculostatic medication given. Particularly the use of PAS might be of importance in this respect. In this manner it might also be possible to explain the difference between SAZU patients and "Zonnegloren" patients, presuming for the time being that intravenous injection of PAS (or isoniazid) gives rise to higher blood level than oral administration of these agents.

The fact that the majority of the tuberculosis patients who showed an unfavourable response to primary immunization subsequently achieved very satisfactory antitoxin titres following a booster injection is in agreement with the behaviour of the immunized guinea-pigs, for which the second Tetanus-P.T. injection can also be regarded as a booster injection.

There is a very exhaustive literature on the influence of various antibiotics (penicillin, chloramphenicol, dihydrostreptomycin, tetracycline, etc.) on immunization after various bacterial infectious diseases, for which we refer to the comprehensive paper by FARI (1959) and the publication of RICHOU and RICHOU (1959).

Patients treated with one of the above-mentioned antibiotics during their illness often showed lower antitoxin titres (agglutinins) than cured patients who were not receiving such medication. The cause of this phenomenon, however, is to be found in the fact that administration of these antibiotics inhibits multiplication of the causative micro-organism, thus giving rise to a quantitatively less marked antigen stimulus. These antibiotics have no demonstrable effect on the RES.

RICHOU and RICHOU (1959), during immunization of experimental animals with diphtheria, tetanus and staphylococcal toxoid, were unable to demonstrate any unfavourable influence of penicillin and streptomycin on the development of antitoxic immunity.

It is not inconceivable therefore, that tuberculostatics such as PAS and isoniazid may behave differently in this respect than the above-mentioned antibiotics.

We propose to verify the possibilities mentioned in this paper in a specifically directed investigation, on which we hope to report in a future publication.

Summary.

The investigation made by WILKENS and TASMAN (1959) into the immunization of tuberculosis patients with Tetanus Phosphate Toxoid has been extended to a further group of patients and control

subjects. The work described here covered a total of 29 controls and 54 tuberculosis patients; the latter were from two different clinics (SAZU and "Zonnegloren").

On the whole, the results previously reported by WILKENS and TASMAN were comfirmed in that tuberculosis patients were found to respond to primary immunization with Tetanus-P.T. (2 injections of 0.5 ml at a one-month interval) by a less marked and slower antitoxin production than the normal controls.

The patients treated at SAZU showed a more deviant behaviour in this respect, than did the "Zonnegloren" patients.

A number of specifically designed animal experiments showed that administration of PAS had an unfavourable effect on development of immunity after the first P.T. injection. This fact might possibly explain the behaviour seen in the tuberculosis patients. It might also explain the marked difference in immunological reactivity between the SAZU and the "Zonnegloren" patients, because the former chiefly received this agent by intravenous drip, while the latter were given oral medication. It is possible that intravenous administration of PAS produces higher blood concentrations than oral administration.

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THE ACETIC ACID BACTERIA, 1941-1961

A CRITICAL REVIEW

by

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INTRODUCTION.

Between the years 1941 and 1961 beliefs regarding the nature of the acetic acid bacteria have changed dramatically several times, and in several different directions. In particular it is to be noted that before 1942 the type of flagellation attracted little, if any, attention, and was apparently not investigated. These bacteria were all included in the single genus *Acetobacter* in the family *Acetobacteriaceae*. After 1942-'43 they seemed universally regarded as having polar flagella; the family *Acetobacteriaceae* was abolished; and in the 6th (1948) edition of BERGEY's Manual the genus *Acetobacter* was consequently placed in the family *Pseudomonadaceae*, this position being still maintained in the 7th (1957) edition.

In 1954, however, LEIFSON claimed that the acetic acid bacteria were of two entirely different types, one peritrichous, and the other polar, this being correlated with the ability and the inability, respectively, to oxidise acetic acid completely to CO_2 and H_2O.

By various groups of workers this finding has been (a) fully confirmed (b) doubted, or only partly accepted and (c) denied altogether. And it has come to our notice that this situation has created a state of utter confusion in the minds of many, to whom it has seemed to be a case of *quot homines tot sententiae*, with no way of knowing (without laborious impartial "bench-work") which of these "opinions" is correct.

It is therefore the purpose of this paper to review critically the position as it has progressed (or retrogressed) over the past twenty years, to record the results of the necessary "bench-work" mentioned, and to try to give to those concerned a clear picture of the true position, which is really remarkably simple.

For this purpose the impact of the various publications on both the taxonomic and the factual position will be discussed, and a clear distinction between those papers which are chiefly devoted to an expression of opinion or criticism, and those which are based factually on valid incontrovertible evidence and reproducible experiments, will be attempted.

REVIEW AND DISCUSSION.

(1) The Position in 1941.

In general this was much as it had been for a very long time, except for the interesting papers of T. ASAI (1934, 1935) – in Japanese. ASAI isolated, from various fruits, 38 strains of bacteria, some of which resembled previously known acetic acid bacteria, whilst others had a somewhat similar oxidative metabolism, particularly towards sugars, notably glucose.

He divided these bacteria into two genera, *Acetobacter* and *Gluconobacter*. The genus *Acetobacter* was then further divided into two sub-genera (A) *Euacetobacter*, which oxidised ethanol but not glucose, and (B) *Acetogluconobacter*, which mainly oxidised ethanol to acetic acid, glucose to gluconic acid "slightly", and mannitol to fructose.

The genus *Gluconobacter* was sub-divided into two sub-genera, (A) *Eugluconobacter*, which oxidised glucose "but not ethanol" and (B) *Gluconoacetobacter*, which mainly oxidised glucose to gluconic acid, and ethanol to acetic acid "slightly".

It will be seen that as *Eugluconobacter* species do not oxidise ethyl alcohol they are not acetic acid bacteria at all. It seems probable that members of this sub-genus were of *Pseudomonas* type. The sub-genus *Gluconoacetobacter*, at first sight, perhaps appears to resemble FRATEUR's "suboxydans group" (which is synonymous with *Acetomonas* Leifson) although the crucial acetate-oxidation is not mentioned. This resemblance, however, cannot be so, because the first *Gluconoacetobacter* species given under this heading is none other than "*Bact. xylinum* Brown", a typical *Acetobacter* species if ever there were one, and the bête noire of the vinegar brewer for its "over-oxidation" of ethanol through acetic acid to CO_2 and H_2O.

It is important to note that these papers of ASAI make no mention of any difference in flagellation type between the various genera and sub-genera in his classification.

(2) R. H. VAUGHN's 1942 and 1943 Papers.

In these two short papers it was claimed that all motile acetic acid bacteria had a "single polar flagellum."

Following this, STANIER (1947) wrote, "In view of the extensive morphological and biochemical parallelism between acetic acid bacteria and organisms of the *P. fluorescens* type, it seems indefensible any longer to maintain a family *Acetobacteriaceae*; its members should be incorporated in the family *Pseudomonadaceae*". This suggestion was adopted in BERGEY's Manual where, in the 6th (1948) edition, *Acetobacteriaceae* was abolished, *Acetobacter* was placed in *Pseudomonadaceae*, and all motile species were stated to have "a single polar flagellum".

(3) FRATEUR's Work.

In 1950 FRATEUR published the results of a largely biochemical study of most available classical species of *Acetobacter*, together with new isolates. For this purpose he used 5 criteria, conformity with which could be determined by simple laboratory tests. These criteria were (1) catalase; (2) "over-oxidation" of ethanol through acetic acid to CO_2 and H_2O; (3) oxidation of lactate to carbonate, which is similar to (2); (4) ketogenic power – notably the oxidation of glycerol to dihydroxyacetone (DHA); (5) the production of gluconic acid from glucose.

According to their reactions to these tests, FRATEUR divided the acetic acid bacteria into 4 groups named, respectively, the Peroxydans, Oxydans, Mesoxydans, and Suboxydans, groups. The last named now corresponds to LEIFSON's genus *Acetomonas*, but in FRATEUR's system its non-acetate-oxidising ability (then not apparently correlated with other unique characters) was regarded as a matter of degree in oxidising power, not as one of the criteria now distinguishing and separating *Acetomonas* strains from *Acetobacter* ones. Species and varieties were separated by using other simple criteria.

(4) Flagellation Type.

The year 1954 was a momentous one for those studying the acetic acid bacteria. For it was then that there were made two major discoveries which were to revolutionize knowledge of these bacteria. It is ironical to note that both were made by workers not interested in the acetic acid bacteria as such.

One of these was the finding, by SCHRAMM and HESTRIN (1954) when studying the mechanism of cellulose-synthesis, that a strain of *Acetobacter xylinum*, when under submerged aeration conditions, gave rise to celluloseless mutants. This later led to the discovery that nearly all *Acetobacter* species were highly mutable (SHIMWELL, 1956 *et seq.*). The other was LEIFSON'S announcement that the acetic acid bacteria comprised two distinct and different types, (a) peritrichously flagellated (*Acetobacter*), and (b) polarly flagellated (*Acetomonas* gen. nov.) and that this was correlated with (a) positive, (b) negative, oxidation of acetate respectively.

From this point onwards this review cannot be strictly chronological, as this would result in a wrong impression of these two subjects being obtained, as this and that worker accepted or denied one or both of the two discoveries mentioned above. First, therefore, it is LEIFSON'S discovery that will be referred to.

The dual flagellation pattern amongst the acetic acid bacteria, claimed by LEIFSON, was not accepted in the 7th (1957) edition of BERGEY'S Manual, as LEIFSON'S photographs were considered inconclusive, and the statement that all motile acetic acid bacteria had "a single polar flagellum" was repeated, pending such time as unequivocal photographs or electron micrographs should be available. Both of these were later published by SHIMWELL (1958 and 1959) but reference to this matter must be deferred to a later stage, for it is now necessary (to maintain continuity of review) to discuss mutation.

(5) Mutation in *Acetobacter*.

SCHRAMM and HESTRIN (1954) were mainly interested in the enzymic mechanism of cellulose synthesis. But it occurred to one of us (J. L. S.) to see whether mutability was confined to their *A. xylinum* strain, or whether other species of *Acetobacter* might show some similar degree of mutability. (It is emphasised throughout this section on mutation that it is the acetate-oxidising genus *Acetobacter* which is referred to, and not *Acetomonas*).

Upon receipt of SCHRAMM and HESTRIN'S parent and mutant strains the latter were found to be, according to FRATEUR'S criteria, *Acetobacter mesoxydans*, having thus lost but the single property of cellulose production (= "a single-step mutation"). All these three strains can be obtained from the N.C.I.B.

The study of many further strains of *Acetobacter*, including fresh isolates and strains from culture collections, showed that almost every strain gave rise to a variety of different colony forms which, when separately cultivated and studied, often proved to be of a "species" different from that indicated by the biochemical reactions of the mass culture as a whole (Shimwell, 1956, 1957a and b, 1959).

Of the many photographs of single microscopic fields taken, 21 were published, the number of different colony forms per single strain varying from 2 to 5. Sometimes the different colony forms (per strain) were present in roughly equal numbers, but more often one type would tend to predominate, the others being present in varying proportions. The results and photographs recorded in these papers resulted in much correspondence from other workers, amongst whom were many who concluded that *Acetobacter* was unique in its rampant mutability, although others wrote to say that the opinion that "species" of *Acetobacter* were unclassifiable (as they usually turned into, or already contained, other "species") was true also of the various genera on which they themselves were specialising.

More recently it has been found (Shimwell and Carr, 1960a) that one *Acetobacter* strain, at least, can give rise to a variety of strange mutants which, when isolated, are found to have lost the power of producing acetic acid from ethanol, whilst retaining many other properties of the parent, thus passing out of the genus *Acetobacter* altogether. These new mutant strains have been given the vernacular name "quasi-acetobacters". If they were isolated from some natural habitat their identity as the parents or progeny of acetobacters would never be suspected.

(6) Flagellation-Type and "Over-oxidation" of Ethanol.

It is now necessary to return to this subject, for it is in connection with the correlation between peritrichous flagellation and acetate-oxidation on the one hand, and absence of such oxidation and polar flagellation on the other, that most of the controversy and confusion has arisen. Shimwell (1958) and, a little later, Asai and Shoda (1958) independently published papers, illustrated with photomicrographs and electron micrographs respectively, fully confirming LEIFSON's claims except in the case of two of ASAI's strains of *Gluconobacter*.

ASAI and SHODA recorded that *Gluconobacter liquefaciens* and *G. melanogenus* had polar flagella and yet over-oxidised ethanol to CO_2 and H_2O. *G. melanogenus* was so named because it produced a dark-brown to black water-soluble pigment which suffused the agar, giving an appearance virtually indistinguishable from that of a culture of *Acetomonas melanogena* (by definition polarly flagellated).

It was nevertheless felt (SHIMWELL and CARR, 1959b) that the flagellation type needed confirmation – partly because these strains were "over-oxidisers", and partly because ASAI and SHODA's two micrographs were not conclusive; in one the "polar" flagellum was not attached to the cell, whilst in the other the several flagella appearing as polar could have originated from off the field of view, as both their ends were not visible.

These two strains were therefore obtained, stained, and found to have the peritrichous flagella necessary for LEIFSON's findings to hold good. In a private communication Prof. ASAI accepted our photograph and findings.

STOUTHAMER (1960), also obtained these strains, and, like us, published a photograph showing peritrichous flagellation. This matter was, moreover, interesting in another way, as on typing *G. melanogenus* by FRATEUR's criteria, it was found to be of the "type" species of *Acetobacter* (*A. aceti*), but a new and unique strain of this on account of its pigment production (CARR and SHIMWELL, 1960).

In the course of another paper STOUTHAMER (1959) also recorded the important finding that whilst *Acetobacter* strains possessed the citric acid cycle, *Acetomonas* ones did not. This to us, appeared to fortify LEIFSON's differentiation, but STOUTHAMER himself took the opposite view, claiming that his results could be taken "to stress the phylogenetic relationship between the four *Acetobacter* groups (and the unity of the whole genus)". "The four groups" referred to are those of FRATEUR.

STOUTHAMER based the above statement on the behaviour of one strain of *Acetobacter mesoxydans* var. *saccharovorans* (FRATEUR's original one) which, although recorded by FRATEUR (1950) as strongly over-oxidising ethanol through acetic acid to CO_2 and H_2O, yet in STOUTHAMER's hands (Warburg technique) failed to oxidise ethanol further than acetic acid. So to him it appeared that, although possessing the citric acid cycle, in other respects it was an *Acetomonas* strain – the very "intermediate", indeed, which, to him, indicated the "unity of the whole genus".

On obtaining this strain, however, SHIMWELL and CARR (1960b) found it to be a strong over-oxidiser of acetic acid, acetate, and lactate. STOUTHAMER (1960, and private communication) later confirmed our findings. So once again LEIFSON's correlation was confirmed.

At this point it is necessary to refer to the work of BROWN and RAINBOW (1956) who, using entirely biochemical methods, found that 28 strains of acetic acid bacteria could be divided into two groups – "lactaphilic" (lactate preferring) and glycophilic (glucose preferring). BROWN and RAINBOW considered that their lactaphilic group might virtually correspond to *Acetobacter* and the glycophilic one to *Acetomonas*.

However, to one of us (J. L. S.) this was not borne out by the species names in BROWN and RAINBOW's lactaphilic group, which included two strains, namely "*A. suboxydans*" NCTC 6430, and "*A. suboxydans*" NCTC 7113, which, from their names, should have been glycophiles. On obtaining and examining these cultures it was indeed found that they were not of the species *A. suboxydans* at all, but were strongly acetate-oxidising acetobacters. On acquainting Dr. RAINBOW with this fact he was gratified to find (private communication) that on transferring these two strains to the lactaphilic group, LEIFSON's *Acetobacter* did correspond to the "lactaphilic" group, and *Acetomonas* to the "glycophilic" one, as he had suggested. Reference to this occurs in a very recent paper (COOKSEY and RAINBOW, 1961).

So once more LEIFSON's findings had been shown correct, and independently fortified by another differentiating criterion – nutritional pattern.

(7) *Acetomonas* and *Pseudomonas*.

Although STANIER's (1947) insistence that the acetic acid bacteria, owing to their alleged universal polar flagellation type and oxidative metabolism, should be transferred to *Pseudomonadaceae* had been refuted by LEIFSON's discovery and its subsequent confirmation, there still remained the question whether the *Acetomonas* strains, being polar, were clearly distinguishable from *Pseudomonas* ones.

SHIMWELL, CARR and RHODES (1960) therefore made a comparative study of 50 strains of *Acetomonas* and 165 strains of *Pseudomonas*. It was found that the two genera could be rapidly and readily differentiated by the use of 4 criteria, (a) production of acid on ethanol/$CaCO_3$/agar oxydogrammes (FRATEUR, 1950), (b) oxidation of calcium lactate to carbonate, (c) production and accumulation of dihydroxyacetone from glycerol, (d) growth at (initial) pH 4.5. *Acetomonas* was positive with (a), (c) and (d), but negative with (b); *Pseudomonas* was positive only with (b). Liquefaction of gelatin, production of greenish-yellow fluorescent pigment, and peptonisation of milk, being variable with *Pseudomonas* and absent from *Acetomonas*, were useful only (as secondary criteria) when positive. One exception to DHA production from glycerol was found amongst acetomonads. Since then another exception has come to light (CARR, 1961), so DHA production really ranks with gelatin liquefaction as a "secondary" characteristic, being useful only when positive.

Nevertheless any two of the above four criteria serve to distinguish, without exception, these two entirely different types of bacteria.

(8) DE LEY'S Views.

One who seems partly to accept the duality of the acetic acid bacteria, but denies the existence of facile mutability in *Acetobacter* is DE LEY (1961). As the result of studying 45 strains of acetic acid bacteria, using "resting" cells suspended in a buffer solution in a Warburg apparatus, he has (a) denied the existence of facile mutability in *Acetobacter* (SHIMWELL, 1956, 1957b, 1958 and 1959); (b) rejected *Acetomonas* in favour of ASAI's name *"Gluconobacter"*; and (c) claimed that these two different genera could arise from a "common pool of ancestors".

As regards facile mutability DE LEY states that he can "not accept SHIMWELL's view". It was not a "view" however, but a long series of published experimental observations and photographs, and it may be that DE LEY's failure to notice mutability was due to the use of submerged "resting cells" in a Warburg apparatus – a technique known to distort the enzymic activity of (the intensely aerobic) acetic acid bacteria as compared with living and growing cells (HROMATKA and EBNER, 1948). If the many factual and photographed observations of SHIMWELL, and SHIMWELL and CARR were doubted, cultures of such mutants were (and are) obtainable for refutation or verification.

As regards rejecting *Acetomonas* in favour of ASAI's "*Gluconobacter*" it will have already been seen, earlier in this review, that ASAI's 1943–45 diagnosis of this was of a mixed genus containing pseudomonads (not oxidising ethanol at all), some possible acetomonads, and some strongly over-oxidising acetobacters, notably *A. xylinum*.

After (N.B. not before) the publication of LEIFSON's (1954) findings, ASAI and SHODA (1958) metamorphosed the 1934–35 diagnosis of *Gluconobacter*, dropping the sub-genera to make it more closely resemble *Acetomonas* Leifson. But their new, simplified, "*Gluconobacter*" was still described as producing little "or no acetic acid from ethanol". Thus *Gluconobacter* still contained an admixture of pseudomonads; and anyway it would surely be taxonomically unjust and invalid to permit, in 1958, a metamorphosis of a 1934–35 diagnosis in order to claim priority over LEIFSON's earlier 1954 publication.

SHIMWELL (1959) found species differentiation in *Acetobacter* impracticable, as when arranged in descending order of possession of positive biochemical properties, each "species" in the list differed from the next by one biochemical property only. It was therefore impracticable to retain individual specific names. Indeed the first in the list (a cellulosic *A. aceti*) might virtually be regarded as the "parent" of all subsequent ones, which could arise by a succession of single-step loss-mutations. SHIMWELL and CARR (1959) found a similar position in *Acetomonas*, and reduced the number of species to one, *Acetomonas oxydans*.

DE LEY (1961) proposes "to regard the entire cluster of strains" of (a) *Acetobacter* and (b) "*Gluconobacter*" respectively as a "biotype" (instead of "genus"), and adopts, as representatives of these two "bio-types", *A. aceti* and *Gluconobacter oxydans*, adding, "It might appear that our proposal to abolish species names for the acetic acid bacteria and to substitute two names of biotypes, is merely a confirmation of the conclusions of SHIMWELL (1959) and SHIMWELL and CARR (1959). In fact, the proposals are fundamentally different and the conclusions were reached on entirely different grounds." We must leave others to judge for themselves whether they consider that there is a difference in this distinction, or whether it is tautological.

As regards De Ley's diagrams showing how *Gluconobacter* and *Acetobacter* could arise "from a common pool of ancestors", he has included in his (non-over-oxidising) "*Gluconobacter* biotype" two strains of strongly over-oxidising *Acetobacter* species, *G. melanogenus* and *G. liquefaciens*, these being the very ones reinvestigated by Stouthamer (1960) and Shimwell and Carr (1960) who all published photographs of the peritrichous flagella. Dr. Leifson also obtained these strains and found them to be "definitely peritrichous" (private communication). Although De Ley states that he had read these two publications, and had seen the photographs, he says, "Pending further investigations, we accept Asai's view". This, of course, refers to Asai's original view, before we and Stouthamer showed, and Asai accepted, that these strains were not polar but peritrichous. It is not stated why this all-important point was not settled by flagella staining these strains while they were actually under study by De Ley.

But when these peritrichous over-oxidisers are removed from their wrong place in the "*Gluconobacter* biotype" diagram, and are transferred to their proper one in the "*Acetobacter* biotype" diagram, then the two diagrams, side by side, do not now suggest a "common pool of ancestors" but, on the contrary, clearly show the sharp and fundamental phylogenetic difference.

(9) The Views of Loitsianskaya and Safronova.

Two papers completely denying peritrichous flagellation amongst the "over-oxidising" acetic acid bacteria, and still insisting that all acetic acid bacteria are polarly flagellated, have come to our notice. These are by the collaborative Russian workers, M. S. Loitsianskaya and L. E. Safronova (1959, 1960) – in Russian.

The first paragraph of their own English final (1960) summary is as follows: –

"Polar attachment of the flagella in motile acetic acid bacteria, as well as the ability of active oxidation transformation and of growth in acid media, indicate that all acetic acid bacteria belong to one genus, *viz. Acetobacter*. Hence the division of this genus into two genera – *Acetomonas* and *Acetobacter*, as suggested by Leifson, has no ground".

In the other (1959) paper there is a large photomicrograph where, amongst about 200 cells on the field of view, two show flagella. One of these cells appears to have a single polar flagellum, the other a single sub-terminal one. This strain was stated to be polar, but yet to be an over-oxidising *Acetobacter*. Upon request, these workers kindly forwarded two similar cultures labelled "*A. aceti*".

Biochemically this name was found to be correct (in respect of FRATEUR's criteria). Both cultures were flagella stained and both were peritrichously flagellated. Fig. 1 shows the result obtained with one of these cultures.

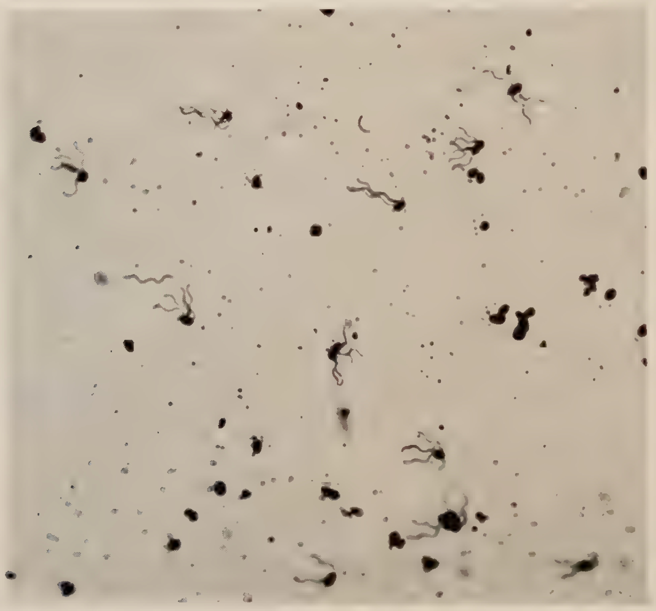

Fig. 1. *A. aceti*, Russian strain. × 1400.

APPENDIX.

Note on Flagella-Staining Acetic Acid Bacteria.

In the course of our work on these bacteria we have had to request cultures described in various papers where the claimed polar flagellation was not in accordance with LEIFSON's correlation with biochemical activity. Almost every case was a claim of polar flagellation which ought to have been peritrichous.

We have always succeeded in flagella-staining these contentious cultures, but have encountered a difficulty which may account for peritrichous strains having been sincerely believed, by others, to be polar ones. Leifson (1954) said that *Acetobacter* strains are more difficult to flagella-stain than most other bacteria. We have not found this to be so in respect of the actual staining of the flagella themselves. What we have found difficult is to avoid detaching them. Fig. 1, for instance, shows a detached flagellum and a fragment of another. With some strains it is not uncommon to find few cells on the slide retaining their flagella at all, even after using the utmost gentleness in the preparation of the film. But many detached loose flagella can then, almost always, be found in great numbers, adrift between the cell bodies.

Also the word "peritrichous" seems nowadays to be often wrongly interpreted, being taken to mean solely "lateral", whereas it really means "all round the periphery". A peritrichously well-flagellated cell, stained without detaching the flagella, will often be seen to have flagella sprouting from all parts of the cell – laterally, subterminally, and also even from, or near, a pole. If, on the other hand, most of the flagella of nearly all the cells on the slide have become detached, then here and there one may find a cell with an apparently polar flagellum (having shed the others). And this may be taken to indicate that the strain is a polarly flagellated one. As we have always found allegedly polar *Acetobacter* strains to be peritrichous, this seems almost the only explanation of the original wrong description. Personally we never describe a culture as "peritrichous" unless many of the cells on the slide have retained their flagella peritrichously, although, in *Acetobacter*, these flagella may be sparse.

Electron Micrographs.

In our experience these, though impressive, are often really less convincing than a photomicrograph showing 7 or 8 or more flagellated cells. Electron micrographs are of such large magnification that there is often only one cell per field, when a single polar flagellum may be of no more significance than as described above in the preceding section. An electron micrograph of a single peritrichous cell, unless it shows both ends of at least some lateral flagella, may also be an artefact, the apparent flagella originating from "off-field" cells, and not from the single central cell in the field at all. Hodgkiss (1960) has, in part, also pointed out the above facts.

Differentiating Criteria.

Leifson's (1954) differentiation (correlation of peritrichous flagella with acetate-oxidation; and polar flagella with non-acetate-oxidation) having been subsequently strongly fortified, the position now is as follows (Table 1):

TABLE 1.

Properties of *Acetobacter* and *Acetomonas*.

	Acetobacter	*Acetomonas*
Ethanol oxidised to $CO_2 + H_2O$	+	—
Lactate oxidised to carbonate	+	—
Nutrition type	Lactaphilic	Glycophilic
Oxidise various amino acids	+	—
Citric acid cycle	+	—
Flagella (if motile)	Peritrichous	Polar

Summary.

Leifson's (1954) differentiation of the acetic acid bacteria into (1) *Acetobacter* – peritrichously flagellated and acetate oxidising, and (2) *Acetomonas* – polarly flagellated and acetate non-oxidising, has, by various workers, been (a) fully confirmed, (b) doubted, or only partly accepted, and (c) denied altogether. Also the priority of *Acetomonas* over *Gluconobacter* has been questioned, as has also facile mutability in *Acetobacter*.

By a critical comparative review of all the publications concerned, and by the experimental examination of the various cultures claimed to contravene Leifson's correlation, we have attempted to clarify the confusion, and to give a clear picture of the true position, which is really quite simple.

The result of so doing has been to vindicate Leifson's findings in every case, to refute criticism of facile mutability in *Acetobacter*, and to show why *Gluconobacter* Asai cannot claim priority over *Acetomonas* Leifson.

Addendum.

Since the above review was written a paper has been published (Joubert, Bayens, and De Ley, 1961) in which these workers, by studying the amino-acid catabolism of the acetic acid bacteria, have unwittingly greatly strengthened LEIFSON's differentiation.

They adopt DE LEY's conception of *A. aceti* as the "biotype" of *Acetobacter*, and *Gluconobacter oxydans* as the biotype of *Gluconobacter*. They record that "All strains from the biotype *Acetobacter aceti* and the acetate-oxidising strains of the biotype *Gluconobacter oxydans*" oxidise a number of amino acids. Those strains "of the biotype *Gluconobacter oxydans* which are unable to oxidise acetate (the suboxydans group according to FRATEUR) are likewise unable to oxidise, deaminate, and to assimilate any amino acid supplied".

But one sees that the very strains of "*Gluconobacter*" which do oxidise amino acids are those which have been shown independently by SHIMWELL, CARR, STOUTHAMER, and LEIFSON (see previously) to be typical peritrichously flagellated acetate-oxidising *Acetobacter* strains. If only these workers would flagella-stain these strains they would see that this is so, and would have to remove these strains from *Gluconobacter* to *Acetobacter*, as we have previously pointed out in this review.

And then their work would be found to constitute, not only further support for LEIFSON's differentiation, but the most weighty support of all. For then the dividing line becomes sharper than ever; *Acetobacter* strains oxidise amino acids: *Acetomonas* ones do not.

We submit that the accumulated evidence shown in Table 1 is beyond refutation.

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ON THE EXISTENCE OF A NEGATIVE PASTEUR EFFECT IN YEASTS CLASSIFIED IN THE GENUS *BRETTANOMYCES* KUFFERATH ET VAN LAER

by

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INTRODUCTION.

Even at a mere glance at the extensive literature on the influence of elementary molecular oxygen on the alcoholic fermentation in living yeast cells and various yeast preparations it is evident that so far most works have been concerned with the inhibiting effect produced by this gas on the rate and extent of the process mentioned. Particularly the relation between the anoxybiontic fermentation and the respiration as measured by the oxygen consumption was and still is one of the main subjects of investigation and speculation. This state of things is confirmed strikingly on studying recent volumes on cellular biochemistry like the "Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto 1957", and the "Ciba Foundation Symposium on the Regulation of Cell Metabolism", which appeared in 1958 and 1959, respectively.

The present paper, on the other hand, deals with the stimulating effect produced by elementary molecular oxygen on the rate of alcoholic fermentation in living cells of yeasts classified in the genus *Brettanomyces* Kufferath et van Laer. If we agree upon the definition of the concept of the "Pasteur effect" given for historical reasons by BURK (1939) as an inhibition of fermentative processes caused by gaseous oxygen, then the phenomenon described in this paper, *i.e.* the inhibition of alcoholic fermentation under strictly anaerobic conditions and its stimulation in presence of molecular oxygen, may be named a "negative Pasteur effect". As a matter of fact, this term was proposed in the sense mentioned as early as in 1940 by CUSTERS in his classical work on the *Brettanomyces* yeasts. When using the term we are, of course, fully aware of the fact that the corresponding physiological effects are not necessarily caused by one and the same mechanism in different yeasts and under different environmental conditions, but rather may be due to a number of mechanisms acting at different steps in the sequence of reactions resulting in breakdown of the sugar molecule to ethyl alcohol and carbon dioxide.

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As mentioned above CUSTERS (1940) discovered a negative Pasteur effect in investigating the alcoholic fermentation of a *Brettanomyces* yeast, *viz. Br. claussenii* Custers. The cells examined by this author by means of the manometric method according to WARBURG originated from plate cultures grown for 3 to 7 days at 30° C. on malt agar supplied with chalk for neutralizing the acetic acid formed (*cf.* below). The harvested cells were washed twice in tap water and then suspended in a 2.5 per cent solution of primary potassium phosphate at pH 4.4 – 4.5. A phosphate solution of the same strength and pH was used in dissolving the glucose to be dissimilated aerobically or anaerobically. Immediately after mixing of cell suspension and glucose solution the sugar concentration was 2 per cent. The total volume of fluid per Warburg vessel amounted to 2.0 ml and the yeast dry substance to 2.72 – 5.92 mg. Anaerobic conditions were obtained by flushing during 20 minutes with nitrogen freed of oxygen by passing over copper gauze at 500 ° C. The sugar dissimilation was measured at 30° C.

Under the experimental conditions summarized briefly above CUSTERS (1940) observed a negative Pasteur effect in cells from 3 days old yeast cultures grown in air, when the cell suspension and glucose solution initially present in the main compartment and side arm of the Warburg vessels, respectively, were mixed after the pretreatment with oxygen-free nitrogen or air for ensuring anaerobiosis and aerobiosis. In examining the fermenting power of cells from cultures grown for 7 days under aerobic conditions CUSTERS, on the other hand, found a normal Pasteur effect, *i.e.* the rate of fermentation in oxygen-free nitrogen surpassed that in air. In these experiments, however, the sugar solution was added to the yeast suspensions previous to the pretreatment with nitrogen or air. According to our experience the results thus obtained with the cells from old cultures, because of the difference in experimental conditions, cannot be compared with those mentioned above for the cells from young cultures. As a matter of fact, no negative Pasteur effect at all or only a minute one was observed by CUSTERS in yeast from young cultures on addition of sugar to the cell suspensions previous to flushing with oxygen-free nitrogen or air.

According to a hypothesis proposed by LIPMANN (1933, 1934, 1942) the normal Pasteur effect, *i.e.* the inhibition of fermentation under aerobic conditions, is due to a reversible oxidative inactivation of an essential part of the glycolytic enzyme system produced by the respiratory enzymes in presence of molecular oxygen. In analogy with this hypothesis CUSTERS (1940) made the assumption that the negative Pasteur effect in the cells of *Br. claussenii* is caused by a reversible reductive inactivation of the proper enzyme system taking place under strictly anaerobic conditions.

In examining the anaerobic and aerobic dissimilation of glucose by proliferating and resting cells of *Br. claussenii* CUSTERS (1940) further showed that ethyl alcohol and carbon dioxide are the only end products formed in nitrogen, while in air in addition appreciable amounts of acetic acid are being produced. In manometric experiments under aerobic conditions with ethyl alcohol as a substrate CUSTERS found that at pH 6.4 – 6.8 the alcohol is oxidized to acetic acid only, whereas at pH 3.8 – 4.4 the primarily formed acetic acid undergoes a complete oxidation to carbon dioxide and water.

The present study was undertaken in order to obtain further information about the existence of a negative Pasteur effect in whole cells of yeasts classified in the genus *Brettanomyces* Kufferath et van Laer according to LODDER and KREGER-VAN RIJ (1952). In addition, we have examined some yeasts which earlier actually were described as *Brettanomyces* species or at least were suspected to be closely related or even belong to this genus but at present are placed in the genera *Hansenula* H. et P. Sydow and *Torulopsis* Berlese. Finally, we tested two yeasts considered as not being definitely or precisely determined, and three yeasts included in the genus *Saccharomyces* (Meyen) Reess and characterized by a comparatively strong production of acetic acid.

MATERIALS AND METHODS.

Yeast strains. So far the following *Brettanomyces* species and strains were examined:

Br. anomalus Custers; two strains: CBS 77 and CBS 3026.

Br. bruxellensis Kufferath et van Laer; four strains: CBS 72, CBS 73, CBS 74 and CBS 96.

Br. bruxellensis Kufferath et van Laer var. *non-membranaefaciens* Custers; four strains: CBS 78, CBS 97, CBS 98 and CBS 3025.

Br. claussenii Custers; two strains: CBS 76 and CBS 1938.

Br. lambicus Kufferath et van Laer; one strain: CBS 75.

Br. schanderlii Peynaud et Domercq (see PEYNAUD and DOMERCQ, 1956); two strains: CBS 2796 and CBS 2797.

Br. vini (Barret, Bidan et André) Peynaud et Domercq (see PEYNAUD and DOMERCQ, 1956); seven strains: CBS 1940, CBS 1941, CBS 1942, CBS 1943, CBS 2336, CBS 2499 and CBS 2547.

For form's sake it may be added that the strains *Br. bruxellensis* CBS 72 and CBS 74, *Br. bruxellensis* var. *non-membranaefaciens* CBS 78, and *Br. lambicus* CBS 75 according to VAN DER WALT and VAN KERKEN (1959a) should be united into a single species, *viz. Br. bruxellensis*. The same authors, furthermore, point out the close relationship of the strain *Br. bruxellensis* CBS 73 to the strain *Br. vini* CBS 1943 and to a strain *Br. vini* Bordeaux 48 which probably is identical with our strain *Br. vini* CBS 2499. These three strains are brought into one species, *Br. intermedius* Krumbholz et Tauschanoff (nov. comb.).

Of the yeasts which earlier were included in the genus *Brettanomyces* or suspected to be closely related to this genus but at present are brought into the genera *Hansenula* and *Torulopsis* the following were tested:

Hansenula anomala (Hansen) H. et P. Sydow; one strain: CBS 1690. This strain was originally obtained from Kufferath in 1927 under the name of *Willia lambica* Kufferath. It was then listed in the catalogue of CBS for 1931 and described by BEDFORD (1942) as *Hansenula lambica* (Kufferath) Dekker. BEDFORD pointed out (p. 640) that the characteristics of this species agree with those given by CUSTERS (1940) for *Brettanomyces lambicus*. However, LODDER and KREGER-VAN RIJ (1952, p. 257) on basis of a comparative study of both organisms state that these cannot belong to one and the same species. By way of example, the former strain shows normal growth on malt agar and may be successfully transferred to a new substrate after a period of six months, whereas the latter organism shows the typical *Brettanomyces* properties of slow growth on malt agar and early death of the cells because of vigorous production of acetic acid. For these and other reasons Lodder and Kreger-van Rij classify the strain CBS 1690 as *Hansenula anomala.*

Torulopsis anomala Lodder et Kreger-van Rij; one strain: CBS 1731. This strain was isolated from a high-salt cucumber brine during the latter stage of fermentation by Etchells and Bell (1950) and was considered by these authors a variety of *Brettanomyces versatilis* Etchells et Bell (*cf.* Lodder and Kreger-van Rij, 1952, p. 431).

To. bacillaris (Kroemer et Krumbholz) Lodder; five strains: CBS 843, CBS 1713, CBS 1779, CBS 2649 and CBS 2799. Of these strains CBS 843 was isolated and described by Kroemer and Krumbholz (1931) and Krumbholz (1931) as *Saccharomyces bacillaris.* The yeast CBS 1713 was obtained from Verona as an authentic strain of the species *Brettanomyces italicus* described by Verona and Florenzano (1947). In studying this organism Lodder and Kreger-van Rij (1952, pp. 423–425) could not confirm the observations reported by the Italian authors in the description and diagnosis of *Br. italicus* with the exception of the property that the cultures lose viability earlier than most other yeasts.

To. etchellsii Lodder et Kreger-van Rij; four strains: CBS 1750, CBS 1751, CBS 2853 and CBS 2854. Of these strains CBS 1750 and CBS 1751 were isolated from high-salt cucumber brines during the latter part of fermentation by Etchells and Bell (1950) and named *Brettanomyces sphaericus.* On having studied the properties of these strains carefully Lodder and Kreger-van Rij (1952, pp. 431 and 428–429) came to the conclusion that they hardly show any relation to the genus *Brettanomyces.*

To. versatilis (Etchells et Bell) Lodder et Kreger-van Rij; nine strains: CBS 1752, CBS 1753, CBS 1754, CBS 1755, CBS 1756, CBS 1757, CBS 1758, CBS 1759 and CBS 1760. The first eight strains (CBS 1752–1759) were isolated by Etchells and Bell (1950) from cucumber brines of extremely high salt strength during the latter stage of fermentation. They were included by the American authors in the genus *Brettanomyces* as a new species named *Br. versatilis.* The results of a careful study of the properties of these strains induced Lodder and Kreger-van Rij (1952, pp. 428–430) to remove them from the genus *Brettanomyces* and include them in the genus *Torulopsis* as *To. versatilis*.

In addition, *Torulopsis apicola* Hajsig, *viz.* strain CBS 2868, isolated from the intestinal tract of a bee and described by Hajsig (1958), and *To. stellata* (Kroemer et Krumbholz) Lodder, *viz.* strain CBS 157, isolated from a grape must with a high sugar concentration (*cf.* Lodder and Kreger-van Rij, 1952, pp. 420–422), were examined.

The following two yeasts were tested for presence of a negative Pasteur effect because of their questionable classification:

Unnamed variety of *Brettanomyces sphaericus* Etchells et Bell; three strains: CBS 1887, CBS 1888 and CBS 1889. These strains were isolated from fermenting cucumber brines and provisionally considered as a variety of *Br. sphaericus* by Etchells and Bell (1952). As mentioned above, this species is classified at present as *Torulopsis etchellsii* (Lodder and Kreger-van Rij, 1952).

Torulopsis cylindrica Walters; one strain: CBS 1947. The strain was isolated from infected beer and described by Walters (1943; see also Walters and Thiselton, 1953). According to this author the strain must be regarded as an exceedingly virulent beer-disease organism capable of causing complete spoilage of the beer when present in the pitching yeast even in an amount of only 0.001 per cent.

As representatives for yeasts, which are not classified in the genus *Brettanomyces* but nevertheless produce comparatively large amounts of acetic acid, the following *Saccharomyces* species were included in our examination:

Sa. aceti Santa María; three strains: CBS 4054, CBS 4070 and CBS 4071. These strains were isolated by Santa María (1958) from red wine of Spanish origin. They are capable of forming a film or pellicle on wine and other liquid media supplied with ethyl alcohol as well as on grape must which has undergone alcoholic fermentation.

Sa. acidifaciens (Nickerson) Lodder et Kreger-van Rij; one strain: CBS 749. This strain was isolated from a bottle of domestic red wine turning sour, and described by Nickerson (1943) as *Zygosaccharomyces acidifaciens*.

Sa. oxidans Santa María; three strains: CBS 4079, CBS 4092 and CBS 4093. Santa María (1958) isolated these strains from a white wine of Spanish origin. Like the strains of *Sa. aceti* mentioned above they are able to form a film or pellicle on wine or grape must after fermentation.

As regards the origin and properties of the yeast strains listed above but not commented upon we refer to LODDER and KREGER-VAN RIJ (1952) and to the Yeast Division, Centraalbureau voor Schimmelcultures, Laboratory of Microbiology, Delft.

Culture media. In growing the yeasts mentioned the following liquid substrates were used:

Medium I and Medium II contained the same amounts of sugar and minerals, *viz.*

Glucose	50.0 g
$(NH_4)_2SO_4$	6.0 g
KH_2PO_4	2.0 g
$MgSO_4 \cdot 7H_2O$ and $CaCl_2$	0.25 g each
H_3BO_3, $ZnSO_4$, $MnCl_2$ and Tl_2SO_4	1.0 mg each
$FeCl_3$	0.5 mg
$CuSO_4$ and KI	0.1 mg each
Distilled water to make	1000.0 ml

The pH value of the media was adjusted to 6.2 – 6.4. In addition Medium I was supplied with 25 μg of biotin and 0.5 mg of thiamin per 1000 ml (*cf.* PEYNAUD and DOMERCQ, 1956), and Medium II with 25 μg of biotin, 0.5 mg of thiamin, 0.5 mg of pyridoxin, 2.5 mg of pantothenic acid (calcium salt), 2.5 mg of riboflavin, 25 mg of meso-inositol, and 100 mg of niacin per 1000 ml. For details in the preparation of these media we refer to WIKÉN and RICHARD (1951, pp. 212–213, nutrient solution B).

Medium III contained 2 per cent glucose in yeast water (prepared from 200 g of pressed baker's yeast and 1000 ml of tap water) and had an initial pH value of 6.2 – 6.4.

The yeast cells used in the fermentation experiments were grown in shake cultures in 100 ml Florence flasks containing 25 ml of one of the liquid media mentioned. These were inoculated with cells raised on malt agar slants which were supplemented with chalk for neutralization of the acid or acids produced. All cultures were kept at 30° C. For the shake cultures the time of incubation varied from 16 to 90 hours (see the proper tables). The cells were harvested by centrifugation at 3000 rpm, washed three times in distilled water and resuspended in fresh distilled water.

Manometric methods. The suspensions of "resting" yeast cells prepared as described above were examined for fermentation power by means of standard Warburg manometric techniques (UMBREIT *et al.*, 1957). The Warburg vessels contained 1.8 ml of cell suspension, corresponding to 20 mg of wet yeast, in the main compartment and 0.2 ml of 1.0 M solution of glucose in distilled water in the side arm. In measuring the oxygen uptake the carbon dioxide liberated was absorbed in 0.2 ml of 20 per cent potassium hydroxide placed in the center cup, which, in addition, contained a roll of analytical grade filter paper for increasing the surface of the alkali.

Anaerobic conditions were obtained in the Warburg vessels by flushing with purified cylinder nitrogen during 30 minutes previous to addition of the sugar to the yeast suspensions. In the earlier experiments this nitrogen was freed of oxygen by passing through a strongly alkaline pyrogallol solution (see KÜSTER, 1921, p. 71; WIKÉN and RICHARD, 1953a, p. 282; METZGER and MÜLLER, 1959, p. 332), and an alkaline solution of sodium hyposulphite (dithionite, hydrosulphite) containing sodium anthraquinone-β-sulphonate as a catalyst (see FIESER, 1924; VOGEL, 1951, p. 182; 1957, p. 186; REICH and KAPENEKAS, 1957; METZGER and MÜLLER, 1959, p. 333). In later experiments the traces of molecular oxygen present in the cylinder nitrogen were removed by passage at room temperature over a catalyst containing finely divided palladium deposited uniformly on purified and finely shredded analytical grade asbestos fibers (see ZELINSKY and BORISOFF, 1924; WINKLER and BRUNCK, 1919, p. 190; 1927, p. 171). Before use the palladium catalyst was saturated with gaseous hydrogen.

Aerobic conditions were obtained by shaking in air during 30 minutes before mixing the sugar solution and yeast suspension present in the side arm and main compartment of the Warburg vessels, respectively.

All Warburg experiments were performed at a temperature of 30° C. The fermentations as well as the respirations were generally run in duplicate or triplicate. In the tables the symbols M and a are used to indicate the mean values and mean deviations, respectively.

In comparing the rates of alcoholic fermentation under anaerobic and aerobic conditions, respectively, the respiration in the sense of an actual uptake of elementary molecular oxygen (O_2) with incomplete or complete oxidation of the sugar or of the ethyl alcohol, formed in fermentation, must be taken into consideration. For the purpose of a discussion some definitions will be given in order to avoid confusion. This is necessary in view of the fact that the meaning of the terms concerned has changed in course of time and still differs with various authors and investigators.

The term respiration is, as mentioned above, here confined to mean the uptake of gaseous oxygen in oxidation of a substrate, whereas the term fermentation is used for a substrate transformation occurring without uptake of this gas. Metabolic processes taking place in presence of molecular oxygen are called aerobic, whether or not the oxygen is being utilized in the reactions concerned, while processes taking place in absence of this gas are named anaerobic. The term oxybiontic (oxybiotic) is applied to transformations accompanied by uptake of molecular oxygen. Processes occurring without employing gaseous oxygen are called anoxybiontic (anoxybiotic). Thus the last two terms are used here to cover the dissimilation reactions distinguished by numerous authors as oxidative and anoxidative or fermentative processes, respectively (*cf.* PASTEUR, 1876; WEINLAND, 1906; KLUYVER, 1924; KLUYVER and DONKER, 1925, 1926; BERNHAUER, 1939, pp. 14–15; STEPHENSON, 1939, p. 16; WERKMAN and WOOD, 1942, pp. 25–26; PORTER, 1946, pp. 794–795 and 896–897; MCBEE, LAMANNA and WEEKS, 1955).

In consequence of the above definitions a fermentation is an anoxybiontic process which may take place under anaerobic as well as aerobic conditions, whereas respiration is an oxybiontic process and thus may occur only under aerobic conditions. We will recur to these definitions elsewhere in connection with a discussion of the mechanism of the negative Pasteur effect in *Brettanomyces* and *Saccharomyces* yeasts.

Calculation of the results. In evaluating the results obtained in the manometric experiments on aerobic sugar dissimilation the following alternatives may be distinguished:

1. The non-proliferating yeast cells do not show any respiration but only fermentation according to the well-known formula:

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2\ CH_3\,.\,CH_2OH$$

If h^2 represents the change in the manometer reading (in mm) of the flask without alkali in the center cup and $k^2{}_{CO_2}$ the flask constant for carbon dioxide, then we get the amount of this gas formed in aerobic fermentation as follows:

$$\mu l\ CO_2{}^{ferm} = h^2 \times k^2{}_{CO_2} \qquad (1)$$

This expression may, of course, also be used when the rate of respiration is negligible as compared to that of fermentation.

2. The yeast cells show marked respiration in addition to fermen-

tation, the end products of aerobic dissimilation being carbon dioxide, ethyl alcohol, acetic acid and water according to the following formulae:

$$C_6H_{12}O_6 \rightarrow 2\,CO_2 + 2\,CH_3\,.\,CH_2OH$$
$$n\,CH_3\,.\,CH_2OH + n\,O_2 \rightarrow n\,CH_3\,.\,COOH + n\,H_2O$$

Combining of the two formulae results in the expression:

$$C_6H_{12}O_6 + n\,O_2 \rightarrow 2\,CO_2 + (2 - n)\,CH_3\,.\,CH_2OH + n\,CH_3\,.\,COOH + n\,H_2O$$

In this expression the value of n may vary between 0 and 2. If n is set equal to 0, the formula for alternative 1 (see above) is obtained, and if n equals 2, the expression assumes the same form as results on substituting 0 for n in the over-all formula for alternative 3 (see below).

If h^1 is the change in the manometer reading of the flask with alkali in the center cup and $k^1{}_{O_2}$ is the constant of the flask for oxygen, then the amount of this gas taken up in respiration is obtained as follows:

$$\mu l\ O_2{}^{resp} = h^1 \times k^1{}_{O_2}$$

From the formulae it appears that carbon dioxide is not formed in respiration but originates entirely from fermentation. Hence:

$$\mu l\ CO_2{}^{resp} = 0$$

Further, the observed change in the manometer reading of the flask without alkali in the center cup, h^2, results from the decrease in pressure due to oxygen uptake in respiration, $h^2{}_a$, and the increase in pressure due to carbon dioxide formation in fermentation, $h^2{}_b$. If $k^2{}_{O_2}$ and $k^2{}_{CO_2}$ are the constants of this flask for oxygen and carbon dioxide, respectively, we get:

$$h^2{}_a = \frac{\mu l\ O_2{}^{resp}}{k^2{}_{O_2}} = h^1 \times \frac{k^1{}_{O_2}}{k^2{}_{O_2}}$$

and

$$h^2{}_b = \frac{\mu l\ CO_2{}^{ferm}}{k^2{}_{CO_2}}$$

Thus:

$$h^2 = h^2{}_b - h^2{}_a = \frac{\mu l\ CO_2{}^{ferm}}{k^2{}_{CO_2}} - \frac{\mu l\ O_2{}^{resp}}{k^2{}_{O_2}}$$

or

$$h^2 \times k^2{}_{CO_2} = \mu l\ CO_2{}^{ferm} - \mu l\ O_2{}^{resp} \times \frac{k^2{}_{CO_2}}{k^2{}_{O_2}}$$

and

$$\mu l\ CO_2^{ferm} = h^2 \times k^2_{CO_2} + \mu l\ O_2^{resp} \times \frac{k^2_{CO_2}}{k^2_{O_2}}. \qquad (2)$$

This equation may, on the assumptions stated above, be applied in calculating the actual amount of carbon dioxide formed in aerobic fermentation.

3. The yeast cells show marked respiration as well as fermentation with formation of carbon dioxide, acetic acid and water as end products according to the formulae:

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2\ CH_3 . CH_2OH$$
$$2\ CH_3 . CH_2OH + 2\ O_2 \rightarrow 2\ CH_3 . COOH + 2\ H_2O$$
$$\frac{n}{2}\ CH_3.COOH + n\ O_2 \rightarrow n\ CO_2 + n\ H_2O.$$

Combining of the three formulae yields the expression:

$$C_6H_{12}O_6 + (2 + n)\ O_2 \rightarrow (2 + n)\ CO_2 + \left(2 - \frac{n}{2}\right) CH_3 . COOH + (2 + n) H_2O.$$

In this expression the value of n may vary in the range of 0 to 4. If n is equal to 0, the expression is identical with that obtained on substituting 2 for n in the over-all formula for alternative 2 (see above). In the case n equals 4, the expression is formally the same as that representing cells with only respiration resulting in complete oxidation of the sugar to carbon dioxide and water:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O.$$

If h^1 is the change in the manometer reading and $k^1_{O_2}$ the flask constant for oxygen, then the volume of this gas taken up in respiration in the Warburg vessel with alkali in the center cup may, as in alternative 2, be calculated as follows:

$$\mu l\ O_2^{resp} = h^1 \times k^1_{O_2}.$$

From the formulae it is evident that carbon dioxide is formed not only in fermentation but also in respiration. The volume of the gas produced in the last process is obtained from the following expression:

$$\mu l\ CO_2^{resp} = \mu l\ O_2^{resp} \times \frac{n}{2 + n} = h^1 \times k^1_{O_2} \times \frac{n}{2 + n}.$$

The change in the manometer reading observed for the vessel without alkali in the center cup, h^2, results from the decrease in pressure due to the uptake of oxygen in respiration, h^2_a, and the increase in pressure due to the carbon dioxide formation in fermentation, h^2_b, and in respiration h^2_c. If $k^2_{O_2}$ and $k^2_{CO_2}$ are the flask constants for oxygen and carbon dioxide, respectively, these three quantities may be expressed as follows:

$$h^2_a = \frac{\mu l\ O_2^{resp}}{k^2_{O_2}} = h^1 \times \frac{k^1_{O_2}}{k^2_{O_2}}$$

$$h^2_b = \frac{\mu l\ CO_2^{ferm}}{k^2_{CO_2}}$$

and

$$h^2_c = \frac{\mu l\ CO_2^{resp}}{k^2_{CO_2}} = \frac{\mu l\ O_2^{resp}}{k^2_{CO_2}} \times \frac{n}{2+n} = h^1 \times \frac{k^1_{O_2}}{k^2_{CO_2}} \times \frac{n}{2+n}.$$

Thus:

$$h^2 = h^2_b + h^2_c - h^2_a = \frac{\mu l\ CO_2^{ferm}}{k^2_{CO_2}} + \frac{\mu l\ O_2^{resp}}{k^2_{CO_2}} \times \frac{n}{2+n} - \frac{\mu l\ O_2^{resp}}{k^2_{O_2}}$$

or

$$h^2 \times k^2_{CO_2} = \mu l\ CO_2^{ferm} + \mu l\ O_2^{resp} \times \frac{n}{2+n} - \mu l\ O_2^{resp} \times \frac{k^2_{CO_2}}{k^2_{O_2}}$$

and

$$\mu l\ CO_2^{ferm} = h^2 \times k^2_{CO_2} + \mu l\ O_2^{resp} \times \left(\frac{k^2_{CO_2}}{k^2_{O_2}} - \frac{n}{2+n}\right). \qquad (3)$$

This expression may, on the assumptions made in alternative 3, be used in calculating the actual volume of carbon dioxide produced in aerobic fermentation.

4. The yeast cells show on one hand fermentation, on the other hand respiration according to the formulae:

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2\ CH_3 . CH_2OH$$

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O.$$

The end products of aerobic dissimilation are thus carbon dioxide, ethyl alcohol and water.

If h^1 is the change of the manometer reading of the Warburg vessel with alkali in the center cup and $k^1_{O_2}$ is the constant of the vessel for oxygen, then the amount of this gas absorbed in respiration is obtained as follows:

$$\mu l\ O_2^{resp} = h^1 \times k^1_{O_2}.$$

From the formulae it appears that carbon dioxide is produced in respiration as well as in fermentation. The volume of this gas formed in the first process is equal to the volume of oxygen taken up:

$$\mu l\ CO_2^{resp} = \mu l\ O_2^{resp} = h^1 \times k^1_{O_2}.$$

The change in the manometer reading found for the flask without alkali in the center cup, h^2, results, as in alternative 3, from the decrease in pressure due to oxygen uptake in respiration, $h^2{}_a$, and the increase in pressure due to carbon dioxide production in fermentation, $h^2{}_b$, and in respiration, $h^2{}_c$. If $k^2{}_{O_2}$ and $k^2{}_{CO_2}$ are the constants of the flask concerned for oxygen and carbon dioxide, respectively, we get:

$$h^2{}_a = \frac{\mu l\ O_2^{resp}}{k^2{}_{O_2}} = h^1 \times \frac{k^1{}_{O_2}}{k^2{}_{O_2}}$$

$$h^2{}_b = \frac{\mu l\ CO_2^{ferm}}{k^2{}_{CO_2}}$$

and

$$h^2{}_c = \frac{\mu l\ CO_2^{resp}}{k^2{}_{CO_2}} = \frac{\mu l\ O_2^{resp}}{k^2{}_{CO_2}} = h^1 \times \frac{k^1{}_{O_2}}{k^2{}_{CO_2}}.$$

Thus:

$$h^2 = h^2{}_b + h^2{}_c - h^2{}_a = \frac{\mu l\ CO_2^{ferm}}{k^2{}_{CO_2}} + \frac{\mu l\ O_2^{resp}}{k^2{}_{CO_2}} - \frac{\mu l\ O_2^{resp}}{k^2{}_{O_2}}$$

or

$$h^2 \times k^2{}_{CO_2} = \mu l\ CO_2^{ferm} + \mu l\ O_2^{resp} - \mu l\ O_2^{resp} \times \frac{k^2{}_{CO_2}}{k^2{}_{O_2}}$$

and

$$\mu l\ CO_2^{ferm} = h^2 \times k^2{}_{CO_2} + \mu l\ O_2^{resp} \times \left(\frac{k^2{}_{CO_2}}{k^2{}_{O_2}} - 1\right). \tag{4}$$

On the assumptions made in alternative 4, this expression may be applied in calculating the actual amount of carbon dioxide formed in aerobic fermentation. Under the experimental conditions applied the value of the quotient between the flask constant for carbon dioxide and that for oxygen varies from 1.072 to 1.083. Hence the values for the oxygen uptake in respiration have to be multiplied by a term ranging from 0.072 to 0.083. Consequently, the correction to be applied for respiration in most cases is comparatively small or even negligible (*cf.* Table 7).

RESULTS.

The results obtained in measuring the rate of fermentation in air and in oxygen-free nitrogen and calculating the aerobic fermentation according to formula 1, *i.e.* without any correction for an oxygen uptake, will be seen in Tables 1, 2 and 3. As a matter of fact, manometer readings were made at intervals of 15 minutes in all experi-

TABLE 1.

Fermentation of glucose by suspensions of resting cells of *Brettanomyces* yeasts in distilled water under aerobic (air) and anaerobic (oxygen-free nitrogen) conditions. The values for the aerobic fermentation are calculated according to formula 1 (see p. 409) *i.e.* without correction for respiration.

Organism		Culture at 30° C.		Carbon dioxide production at 30° C. per 20 mg of wet yeast per 90 minutes in µl of 0° C. and 760 mm			
Species	Strain CBS No.	Medium	Age in hours	Aerobically		Anaerobically	
				M	a	M	a
Br. anomalus	77	I	68	87	3	42	1
	3026	I	46	141	0	65	2
Br. bruxellensis	72	II	46	49	1	8	0
	72	I	90	35	1	21	0
	73	I	42	184 a)	4	67 a)	7
	74	I	70	282	0	39	2
	96	I	45	88	4	32	4
Br. bruxellensis var. *non-membranaefaciens*	78	I	68	114	4	36	7
	97	I	69	170	4	40	6
	98	I	42	163	10	82	1
	3025	I	41	162 b)	3	67 b)	1
Br. claussenii	76	I	23	107	4	27	9
	76	I	44	538	21	23	2
	76	I	45	668	4	158	16
	76	I	46	616	7	74	8
	76	I	46	493	5	34	2
	76	I	48	647	40	38	1
	76	III	20	639	0	39	10
	76	III	21	544	18	120	6

Br. schanderlii	2796	I	66	107	3	58	1
	2797	I	69	120	3	62	1
Br. vini	1940	I	43	272 d)	0	63 d)	1
	1941	I	22	355	5	111	2
	1941	I	41	113 e)	1	119 e)	4
	1942	I	22	356	1	27	1
	1942	I	41	331 f)	1	387 f)	0
	1942	I	46	636	25	642	—
	1943	I	22	342	5	73	6
	1943	I	42	223	2	254	6
	1943	I	44	682	11	527	10
	2336	I	42	134	0	28	7
	2499	I	42	189	6	80	2
	2547	I	45	451	1	264	4

M = mean values of duplicates or triplicates
a = mean deviation

a) after 105 minutes 209 μl (a = 3) under aerobic and 81 μl (a = 7) under anaerobic conditions.
b) after 105 and 120 minutes, respectively, 184 μl (a = 2) and 203 μl (a = 3) under aerobic and 79 μl (a = 2) and 93 μl (a = 2) under anaerobic conditions.
c) after 105 minutes 169 μl (a = 1) under aerobic and 61 μl (a = 0) under anaerobic conditions.
d) after 105 minutes 305 μl (a = 0) under aerobic and 76 μl (a = 2) under anaerobic conditions.
e) after 105 and 120 minutes, respectively, 138 μl (a = 1) and 158 μl (a = 1) under aerobic and 149 μl (a = 5) and 180 μl (a = 5) under anaerobic conditions.
f) after 105 and 120 minutes, respectively, 384 μl (a = 3) and 432 μl (a = 4) under aerobic and 475 μl (a = 1) and 553 μl (a = 1) under anaerobic conditions.

TABLE 2.

Fermentation of glucose by suspensions of resting cells of *Hansenula* and *Torulopsis* yeasts in distilled water under aerobic (air) and anaerobic (oxygen-free nitrogen) conditions. The values for the aerobic fermentation are calculated according to formula 1 (see p. 409), *i.e.* without correction for respiration.

Organism		Culture at 30° C.		Carbon dioxide production at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm			
Species	Strain CBS No.	Medium	Age in hours	Aerobically		Anaerobically	
				M	a	M	a
Ha. anomala	1690	I	48	4	2	33	2
To. anomala	1731	I	43	55 a)	7	278 a)	35
To. apicola	2868	I	46	7	4	78	2
To. bacillaris	843	II	18	879	24	1372	12
	843	II	19	719	11	947	6
	1713	II	19	987	13	1501	20
	1779	II	19	1132	26	1272	26
	2649	II	19	798	4	1269	30
	2799	II	19	974	23	1387	12
To. etchellsii	1750	I	18	17	2	13	1
	1750	I	22	20	3	38	0
	1750	I	44	9	1	15	2
	1751	I	20	15	2	155	2
	2853	I	19	20	3	23	1
	2853	I	20	11	2	20	1
	2854	I	19	5	2	24	1
To. stellata	157	III	46	783	30	784	17
To. versatilis	1752	I	44	65	10	430	12
	1753	I	45	57	2	332	3
	1754	I	44	47	2	311	2
	1755	I	44	42	6	263	7
	1756	I	19	36	1	230	3
	1757	I	43	7	3	43	3
	1758	I	19	13	1	181	3
	1759	I	19	23	3	277	5
	1760	I	22	45	6	206	2

M = mean values of duplicates or triplicates
a = mean deviation
a) after 105, 120, 135 and 150 minutes, respectively, 57 μl (a = 7), 59 μl (a = 9), 59 μl (a = 9) and 57 μl (a = 9) under aerobic and 329 μl (a = 41), 390 μl (a = 48), 446 μl (a = 53) and 501 μl (a = 62) under anaerobic conditions.

TABLE 3.

Fermentation of glucose by suspensions in distilled water of resting cells of *Saccharomyces* yeasts and yeasts, classified at present as an unnamed variety of *Brettanomyces sphaericus* and as *Torulopsis cylindrica*, under aerobic (air) and anaerobic (oxygen-free nitrogen) conditions. The values for the aerobic fermentation are calculated according to formula 1 (see p. 409), *i.e.* without correction for respiration.

Organism		Culture at 30° C.		Carbon dioxide production at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm			
Species	Strain CBS No.	Medium	Age in hours	Aerobically		Anaerobically	
				M	a	M	a
Sa. aceti	4054	I	42	266	1	336	7
	4070	I	42	215	3	425	6
	4071	I	21	1085	6	1468	18
Sa. acidifaciens	749	I	21	656	5	1106	18
Sa. oxidans	4079	I	16	787	10	918	4
	4079	I	21	840	24	862	20
	4092	I	16	274	3	359	1
	4093	I	16	854	13	944	17
	4093	I	42	789	18	840	19
Unnamed variety of *Br. sphaericus*	1887	I	46	9	1	59	1
	1888	I	46	11	3	98	1
	1889	I	18	36	4	182	1
To. cylindrica	1947	I	18	186	1	59	4
	1947	I	19	227	3	112	3
	1947	I	42	215	3	51	7

M = mean values of duplicates or triplicates
a = mean deviation

ments, but from considerations of space and clearness in most cases only the values observed after 90 minutes are listed. In a few cases the fermentation was measured even over longer periods than 90 minutes. The values thus obtained are also recorded in the tables mentioned.

From the results presented in Table 1 it is evident that all 22 strains of the *Brettanomyces* species so far tested show a negative Pasteur effect irrespective of the medium used for growing the cells concerned. Furthermore, it is obvious that, on comparing the rate of anaerobic fermentation with the corresponding values for the aerobic process calculated according to formula 1, *i.e.* without consideration of the oxygen uptake, mostly no negative Pasteur effect is found in cells harvested from 41–46 hours old cultures of the strains CBS 1941, CBS 1942 and CBS 1943 of *Br. vini*, whereas in cells from 22 hours old cultures of the same strains a typical effect may be observed.

It may be added that in the cells manifesting a negative Pasteur effect after a period of 90 or more minutes this effect sets in immediately after addition of the glucose solution to the cell suspensions, the fermentation curve obtained under aerobic conditions thus from the very beginning of the sugar dissimilation diverging from that observed under anaerobic conditions.

For the cells from 41 hours old cultures of *Br. vini*, strain CBS 1941, which do not show any negative Pasteur effect, the fermentation curves recorded in air and in oxygen-free nitrogen, respectively, are practically identical up to 90 minutes, whereas the cells from 41, 46 and 42 hours old cultures of *Br. vini*, strains CBS 1942, CBS 1942 and CBS 1943, respectively, show a negative Pasteur effect up to 60, 75 and 60 minutes. Subsequently, however, this effect disappears, and the dissimilation curves cross one another at about 67, 85 and 65 minutes, respectively, the curves for the cell suspensions in air showing a decrease and those for the suspensions in oxygen-free nitrogen an increase in the slope with time.

From the data given in Table 2 it is obvious that no negative Pasteur effect may be demonstrated in *Hansenula anomala* and the 20 strains of the six *Torulopsis* species examined, on comparing the amounts of carbon dioxide formed under anaerobic conditions with those recorded for aerobic conditions without correction for the respiration. It may be mentioned that in these experiments, with a few exceptions, all manometer readings made for the anaerobic cell suspensions are higher than the corresponding readings for the aerobic suspensions, the curve for anaerobic conditions hence from the very beginning of the glucose dissimilation diverging from that obtained for aerobic conditions. In a few *Torulopsis* strains the curves recorded for the anaerobic and aerobic sugar dissimilation, respectively, are practically identical.

The results listed in Table 3 show conclusively that, on calculating the aerobic carbon dioxide formation according to formula 1 and hence neglecting the respiration, no negative Pasteur effect may be observed in the *Saccharomyces* species tested or in the unnamed variety of *Brettanomyces sphaericus*, whereas the cells from 18–42 hours old cultures of *Torulopsis cylindrica* show such an effect of the same order of magnitude as found in typical *Brettanomyces* strains. It may be added that the experimental results are clear-cut, the curves for the aerobic and anaerobic glucose dissimilation diverging promptly from one another from the addition of the sugar on, or in two cases, *viz.* in the cells of the strains CBS 4079 and CBS 4093 of *Sa. oxidans* from 21 and 42 hours old cultures, respectively, showing approximately the same course.

Summarizing the results presented in Tables 1, 2 and 3, we may state that, if the oxygen uptake in respiration is not taken into account in calculating the amounts of carbon dioxide formed in aerobic fermentation, then a negative Pasteur effect can be proved only in the true *Brettanomyces* species, and in the strain of *Torulopsis cylindrica* examined. In all other yeast strains tested, classified as *Hansenula*, *Torulopsis* and *Saccharomyces* species, as well as in the unnamed variety of *Brettanomyces sphaericus*, such an effect cannot be demonstrated on applying formula 1 in calculating the formation of carbon dioxide in aerobic fermentation.

The actual magnitude of the negative Pasteur effect established qualitatively by using formula 1 in estimating the aerobic fermentation may be found by determining the corresponding oxygen uptake in respiration and applying one of the formulae 2, 3 and 4 for calculating the proper correction. The choice of formula has, of course, to be based on a careful examination of the type of oxybiontic sugar dissimilation involved. Experimental work on this problem is in progress in our laboratory. Attention may here be called to the fact that the actual values for the aerobic fermentation are even higher than the values obtained by calculation according to formula 1. On the basis of the data presented in Tables 1 and 3 therefore only minimum values for the magnitude of the negative Pasteur effect in the yeasts concerned may be estimated.

Furthermore, it is *a priori* quite possible that yeast strains in which no negative Pasteur effect is found on calculating the aerobic fermentation without correction for the respiration, nevertheless in reality possess this effect. Such a "masking" of the negative Pasteur effect is to be expected particularly in yeasts with a comparatively high oxygen uptake accompanied by an incomplete oxidation of the ethyl alcohol formed in fermentation.

In order to get some preliminary information about the order of magnitude of the corrections to be applied according to formulae 2, 3 and 4 because of respiration, the oxygen uptake of the yeasts was measured using cells from cultures of approximately the same age as in the determination of the carbon dioxide formation. Some results are listed in Tables 4, 5 and 6. As was to be expected, the yeasts behave in different ways as regards the dependence of the respiration on the age of the cultures concerned, the oxygen uptake in some strains being comparatively constant, in other strains increasing or decreasing with an increase in the culture age. Some yeast strains showed a high reproducibility as regards the respiration in cells from cultures of the same age, whereas in other strains a strong variation was observed in the rate of oxygen uptake of such cells from one experiment to another. In some species approximately the same respiratory activity was observed for the different strains tested, whereas in other species great differences were found between strains as regards the rate of the oxygen uptake even on comparing cells from cultures of about the same age. Anyhow, it is evident from the values listed in Tables 4, 5 and 6 that the respiration in several yeasts is of such an order of magnitude that a masking of an eventual negative Pasteur effect cannot *a priori* be excluded or even seems quite possible. In view of the strong variation in respiratory activity mentioned the oxygen uptake in respiration and the formation of carbon dioxide in anaerobic fermentation as well as the mixed sugar dissimilation under aerobic conditions were measured using portions of one and the same yeast suspension in air in Warburg vessels with alkali in the center cup, and in oxygen-free nitrogen and air, respectively, in vessels without alkali. We are, of course, fully aware of the fact that this "direct method" for measuring the aerobic glucose dissimilation gives correct results only on the condition that the metabolic processes involved go on

TABLE 4.

Glucose respiration of suspensions of resting cells of *Brettanomyces* yeasts in distilled water in air.

Organism		Culture at 30° C.		Oxygen uptake at 30° C. per 20 mg of wet yeast per 90 minutes in µl of 0° C. and 760 mm	
Species	Strain CBS No.	Medium	Age in hours	M	a
Br. anomalus	77	I	65	100	1
	3026	I	42	29	3
Br. bruxellensis	72	II	44	82	1
	73	I	42	92	6
	74	I	67	15	5
	96	I	44	111	0
Br. bruxellensis var. *non-membranaefaciens*	78	I	65	81	7
	98	I	43	77	2
	3025	I	40	89	9
Br. claussenii	76	I	46	19	—
	1938	I	40	136	0
Br. lambicus	75	I	44	136	2
	75	I	65	26	2
Br. schanderlii	2796	I	67	85	2
	2797	I	66	148	—
	2797	I	67	160	—
Br. vini	1940	I	42	120	0
	1941	I	19	131	2
	1941	I	40	104	1
	1941	I	42	41	5
	1942	I	21	116	5
	1942	I	40	125	1
	1942	I	41	153	—
	1943	I	21	155	13
	1943	I	43	70	2
	2336	I	42	86	12
	2499	I	41	80	5
	2547	I	43	59	—
	2547	I	43	87	—

M = mean values of duplicates or triplicates
a = mean deviation

TABLE 5.

Glucose respiration of suspensions of resting cells of *Hansenula* and *Torulopsis* yeasts in distilled water in air.

Organism		Culture at 30° C.		Oxygen uptake at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm	
Species	Strain CBS No.	Medium	Age in hours	M	a
Ha. anomala	1690	I	43	216	3
	1690	I	45	81	13
To. anomala	1731	I	45	312	1
To. apicola	2868	I	46	13	—
To. bacillaris	843	II	18	316	7
	1713	II	18	98	4
	1779	II	16	211	10
	2649	II	18	275	8
	2799	II	16	111	5
	2799	II	16	340	9
	2799	II	18	234	20
To. etchellsii	1750	I	18	234	11
	1751	I	16	184	8
	1751	I	19	113	15
	2853	I	19	193	10
	2854	I	16	218	2
	2854	I	19	187	—
To. stellata	157	III	46	17	—
To. versatilis	1752	I	44	250	4
	1753	I	45	166	5
	1754	I	44	67	5
	1755	I	43	215	1
	1755	I	45	204	—
	1756	I	17	196	—
	1756	I	21	162	17
	1757	I	45	187	7
	1758	I	21	226	2
	1759	I	21	168	2
	1760	I	21	132	1

M = mean values of duplicates or triplicates
a = mean deviation

TABLE 6.

Glucose respiration in air of suspensions in distilled water of resting cells of *Saccharomyces* yeasts and yeasts, classified at present as an unnamed variety of *Brettanomyces sphaericus* and as *Torulopsis cylindrica*.

Organism		Culture at 30° C.		Oxygen uptake at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm	
Species	Strain CBS No.	Medium	Age in hours	M	a
Sa. aceti	4054	I	43	48	10
	4070	I	40	90	1
	4071	I	17	143	5
Sa. acidifaciens	749	I	17	157	9
Sa. oxidans	4079	I	21	51	4
	4092	I	17	11	—
	4093	I	17	23	5
Unnamed variety of *Br. sphaericus*	1887	I	17	68	14
	1887	I	40	143	7
	1888	I	40	374	4
	1889	I	16	151	5
To. cylindrica	1947	I	17	133	4
	1947	I	19	102	—
	1947	I	44	44	7

M = mean values of duplicates or triplicates
a = mean deviation

TABLE 7.

Glucose respiration in air and fermentation in air and in oxygen-free nitrogen by suspensions in distilled water of resting cells of *Brettanomyces* and *Torulopsis* species and of a yeast, classified at present as an unnamed variety of *Brettanomyces sphaericus*. The values for the aerobic fermentation are calculated on one hand without correction for respiration according to formula 1 (see p. 409), on the other hand with correction for this process according to formula 2 (see p. 411), formula 3 for n = 0, 1, 2, 3 and 4 (see p. 412), and formula 4 (see p. 413), respectively.

Organism		Culture at 30° C.		Oxygen uptake at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm	Carbon dioxide production at 30° C. per 20 mg of wet yeast per 90 minutes in μl of 0° C. and 760 mm	
Species	Strain CBS No.	Medium	Age in hours		Aerobically	Anaerobically
Br. claussenii	76	I	46	19	493 (1) 514 (2) 514 507 504 (3) 502 501 494 (4)	34
To. apicola	2868	I	46	13	7 (1) 21 (2) 21 17 15 (3) 14 13 8 (4)	78

					1220 (2) 1220 1115 1062 (3) 1030 1009 904 (4)	
To. etchellsii	1750	I	18	234	17 (1) 269 (2) 269 191 152 (3) 128 113 35 (4)	13
To. stellata	157	III	46	17	783 (1) 801 (2) 801 796 793 (3) 791 790 785 (4)	784
Unnamed variety of *Br. sphaericus*	1887	I	40	143	32 (1) 186 (2) 186 139 115 (3) 101 91 44 (4)	116

at the same rate irrespective of whether carbon dioxide is present in the gas phase above the yeast suspensions or not. Some results obtained in these experiments are presented in Table 7.

On comparing the values given in Table 7 for the amounts of carbon dioxide formed in anaerobic fermentation with those found for the aerobic fermentation by calculation according to formulae 1, 2, 3 and 4 it will be seen that the magnitude of the negative Pasteur effect in strain CBS 76 of *Brettanomyces claussenii* does not change very much on considering the respiration, the oxygen uptake being a rather small one and the carbon dioxide volume produced in aerobic fermentation varying from a minimum value of 493 μl (formula 1; without correction for respiration) to a maximum value of 514 μl (formulae 2 and 3; with correction for respiration; $n = 0$ in formula 3). Furthermore, it is evident from Table 7 that no negative Pasteur effect may be found in strain CBS 2868 of *Torulopsis apicola* and strain CBS 843 of *To. bacillaris* even on application of a maximum correction for the oxygen uptake, the values for the anaerobic fermentation being 78 μl and 1372 μl, and those for the aerobic fermentation varying within the ranges of 7–21 μl and 879–1220 μl of carbon dioxide, respectively. In strain CBS 157 of *To. stellata* the values for the aerobic fermentation obtained on applying corrections for respiration according to formulae 2 and 3, *viz.* 790–801 μl, are somewhat higher than the corresponding value for the anaerobic fermentation, *viz.* 784 μl, but the differences are undoubtedly too small to be considered as significant, the strain hence probably lacking a negative Pasteur effect. In the unnamed variety of *Brettanomyces sphaericus*, strain CBS 1887, a comparatively great difference is obtained between the rate of the aerobic fermentation and that of the anaerobic process on the assumption that it is allowed to apply a maximum correction for the oxygen uptake, the value calculated according to formulae 3 (for $n = 0$) and 2 for the amount of carbon dioxide formed in aerobic fermentation being 186 μl, while the corresponding value for anaerobic conditions is only 116 μl. In view of the fact, however, that on calculation using the other formulae or other values for n, respectively, values for the aerobic fermentation are obtained which are definitely smaller than or practically identical with or, considering the limits of experimental error, not clearly different from the value directly determined for the anaerobic process (32 and 44 μl; 115 μl; 91, 101 and 139 μl; as compared to 116 μl), we would prefer to leave the question whether or not a masked negative Pasteur effect exists in strain CBS 1887 of the unnamed variety of *Br. sphaericus* open until sufficient information about the nature of the oxybiontic glucose dissimilation of this strain is available.

Finally, it is evident from Table 7 that a masked negative Pasteur effect very probably exists in strain CBS 1750 of *Torulopsis etchellsii*. The uncorrected value for the aerobic fermentation, *viz.* 17 μl, is practically identical with the value for the anaerobic fermentation, *viz.* 13 μl, while the latter value is throughout surpassed by the values for the aerobic fermentation found on correction for the respiration according to formulae 2, 3 and 4, *viz.* 269 μl, 113, 128, 152, 191 and 269 μl, and 35 μl, respectively.

It may be mentioned that in all experiments presented in Table 7 manometer readings were made at intervals of 15 minutes. The values for a fermentation and respiration period of 90 minutes, listed in the table, fall throughout on smooth curves.

DISCUSSION AND CONCLUSIONS.

A negative Pasteur effect in the sense of an inhibition of the rate of the alcoholic fermentation under anaerobic conditions (in oxygen-free nitrogen) and a stimulation of this process in presence of molecular oxygen (in air) was conclusively demonstrated in two strains of *Brettanomyces anomalus*, four strains of *Br. bruxellensis*, four strains of *Br. bruxellensis* var. *non-membranaefaciens*, two strains of *Br. claussenii*, one strain of *Br. lambicus*, two strains of *Br. schanderlii*, and seven strains of *Br. vini*, *i.e.* in all *Brettanomyces* strains so far tested. It has to be stressed that these strains represent organisms which have been subcultured on ordinary laboratory media for long periods of time. In view of the variation from type occurring more or less commonly in microbes on keeping them in artificial culture no conclusions may be drawn about the presence or absence of such an effect in freshly isolated *Brettanomyces* strains.

In the *Brettanomyces* strains mentioned the negative Pasteur effect can be proven even in calculating the amounts of carbon dioxide formed in aerobic fermentation without correction for an eventual simultaneous uptake of molecular oxygen and the more or less equivalent formation of carbon dioxide in respiration. This holds for the *Brettanomyces* strains with a comparatively high respiration ($\geqq$ 75–100 μl of O_2 per 20 mg of wet yeast per 90 minutes) as well as for those with a low respiration ($\cong$ 15–30 μl of O_2 per 20 mg of wet yeast per 90 minutes).

In some strains of *Brettanomyces vini* the negative Pasteur effect may be demonstrated only in cells from young cultures on calculating the aerobic fermentation without consideration of the oxygen uptake.

It may be added that the actual values for the negative Pasteur effect obtained on correction for the respiration will be even greater than the values found without correction. This will be the case in particular when the oxygen taken up is used in incomplete oxidation without formation of carbon dioxide, *e.g.* in conversion of the ethyl alcohol, produced in fermentation, to acetic acid.

In addition, a negative Pasteur effect could be established in one strain of *Torulopsis cylindrica* even without applying any correction for the molecular oxygen taken up in respiration ($\cong$ 40–135 μl per 20 mg of wet yeast per 90 minutes).

In all other yeasts so far examined, *viz.* one strain of *Hansenula anomala*, one strain of *Torulopsis anomala*, one strain of *To. apicola*, five strains of *To. bacillaris*, four strains of *To. etchellsii*, one strain of *To. stellata*, nine strains of *To. versatilis*, three strains of *Saccharomyces aceti*, one strain of *Sa. acidifaciens*, three strains of *Sa. oxidans*, and three strains of an unnamed variety of *Brettanomyces sphaericus* (classified at present as *Torulopsis etchellsii*), no negative Pasteur effect could be observed on estimating the aerobic fermentation without correction for respiration. Some of these yeast strains are characterized by a very low, other strains by a moderate to high respiration, the oxygen uptake varying between 11 and 374 μl of O_2 per 20 mg of wet yeast per 90 minutes. It has to be emphasized that most of the strains are included in yeasts which earlier were described as *Brettanomyces* species or were suspected to be closely related to or even belong to this genus, while other strains are similar to *Brettanomyces* yeasts in possessing the capacity to form acetic acid.

In some of the yeast strains which do not show any negative Pasteur effect on calculating the aerobic fermentation without correction for the respiration, the anaerobic fermentation and the mixed aerobic dissimilation (aerobic fermentation + respiration) of glucose was examined using one and the same cell material. On application of a correction for the oxygen uptake in respiration in calculating the carbon dioxide formed in aerobic fermentation according to three alternatives no negative Pasteur effect was found in strains of yeasts like *Torulopsis apicola*, *To. bacillaris* and *To. stellata*. In a strain of the unnamed variety of *Br. sphaericus* such an effect was obtained only on applying the maximum correction for respiration. Hence, a decisive conclusion as regards the existence of a negative Pasteur effect in this yeast has to be postponed until a careful examination of its oxybiontic sugar dissimilation has proven that such a correction actually is legitimate. In a strain of *To. etchellsii*, finally, a negative Pasteur effect was observed on correction for the oxygen uptake according to all alternatives so far considered. On neglecting this correction the effect is being masked due to the uptake of molecular oxygen.

On basis of the above findings the following three groups of yeasts may be distinguished:

1) yeasts possessing a negative Pasteur effect which may be observed by manometric techniques even without application of any correction for the oxygen uptake and carbon dioxide formation in respiration; this group includes all *Brettanomyces* strains so far tested, and, in addition, a strain of *Torulopsis cylindrica*;

2) yeasts possessing a negative Pasteur effect which is masked by the uptake of molecular oxygen in respiration, and, hence, can be proved by manometric techniques only on application of the proper correction for respiration; strains of *Torulopsis etchellsii* are representatives of this group;

3) yeasts lacking a negative Pasteur effect.

As will be reported in detail elsewhere, the negative Pasteur effect in the *Brettanomyces* yeasts may be demonstrated not only in cell suspensions in distilled water, but also in suspensions prepared with phosphate buffer or with solutions of mixtures of organic acids and their sodium or potassium salts, *e.g.* citric acid-citrate, DL-malic acid-malate, fumaric acid-fumarate, succinic acid-succinate, D-tartaric acid-tartrate, malonic acid-malonate, and acetic acid-acetate mixtures. Now WIKÉN and co-workers (1953b, 1954a and b, 1955a and b, 1957, 1958, 1959, 1961) and PFENNIG and WIKÉN (1960a and b) have demonstrated the existence of a negative Pasteur effect in intact cells of strains of *Saccharomyces carlsbergensis*, *Sa. cerevisiae*, and *Sa. uvarum*. This effect, however, is caused specifically by the succinic acid-succinate system, whereas no effect at all or only a minute one is obtained in distilled water, phosphate buffer or solutions of mixtures of other organic acids and their salts. Hence, an additional group of yeasts exists, *viz.*

4) yeasts showing a negative Pasteur effect only under particular environmental conditions, *e.g.* in suspensions of resting cells in succinic acid-succinate solutions of definite concentrations and pH values; in the strains of the *Saccharomyces* species mentioned this effect appears only in cells from very young cultures.

In view of the results described in the present paper it seems quite reasonable to ponder the question whether or not the presence or absence of a negative Pasteur effect could be used as a criterion in classification of yeasts. In this connection it has to be considered that even minute amounts of molecular oxygen may have a stimulating effect on the rate of alcoholic fermentation. By way of example, SCHEFFERS (1961) mentions that a marked increase in the rate of this process is observed in *Brettanomyces claussenii* in presence of 0.1 per cent of oxygen in the gas phase. Still earlier WIKÉN and RICHARD (1955b) have shown that in the Fendant strain of *Saccharomyces carlsbergensis* a stimulation sets in on supplementing the gas phase above the cell suspensions with 0.005 per cent by volume of molecular oxygen. Under the experimental conditions applied this means that even amounts of only 2.5 – 3.0 μl of oxygen, present in the gas phase, are active per 300 mg of wet yeast or 60 mg of yeast dry substance, corresponding on a weight basis to ratios of about 1–1.5 : 100,000 and 1–1.5 : 20,000, respectively. Undoubtedly it is a matter of course that the expediency and legitimacy of using Einhorn or Durham tubes without definitely securing anaerobiosis and aerobiosis in testing yeasts for their ability to ferment different sugars should be the subject of a careful re-examination. Before applying the presence or absence of a negative Pasteur effect as a characteristic in yeast taxonomy critical fermentation tests under on one hand strictly anaerobic, on the other hand strictly aerobic conditions should be carried out with a great number of yeasts belonging to different genera. Work along these lines is now in progress in our laboratory.

For the moment the fact that all *Brettanomyces* strains so far tested possess a negative Pasteur effect which may be proven even without applying any correction for respiration, and that the yeasts at present brought to the genera *Hansenula* and *Torulopsis* but earlier described as *Brettanomyces* species or suspected to be closely related or even belong to this genus, lack such a non-masked effect, may undoubtedly be taken as evidence in support of removing these yeasts from the genus *Brettanomyces*.

The fact that the strain of *Torulopsis cylindrica* Walters possesses a marked non-masked negative Pasteur effect has led to a re-examination of the systematic position of this yeast. The results will be published elsewhere before long.

The existence of a negative Pasteur effect in the sense described in the present paper is of considerable interest in view of the classical conception of the alcoholic fermentation in yeast as an anoxybiontic process which may take place under strictly anaerobic conditions. Custers (1940) as well as van der Walt and van Kerken (1959b) have shown that the anaerobic or "micro-aerobic" glucose dissimilation in *Brettanomyces claussenii* is a normal alcoholic fermentation yielding equimolar amounts of ethyl alcohol and carbon dioxide, and, hence, occurring without consumption of any molecular oxygen. Notwithstanding this fact the alcoholic fermentation in this yeast takes place at a high rate only under aerobic conditions or, under anaerobic conditions, on external addition of oxidized coenzyme I or certain carbonyl compounds like acetaldehyde, acetone and pyruvic acid, which may replace molecular oxygen as hydrogen acceptors (Scheffers, 1961). The molecular oxygen seems to stimulate the anoxybiontic process by partially re-oxidizing coenzyme I and possibly also other enzyme components which, under the experimental conditions applied, have been reduced too far (*cf.* Custers, 1940; Kluyver and Custers, 1940; Wikén and Richard, 1953b, 1954a and b, 1955a and b; Pfennig and Wikén, 1960a and b).

Summary.

The present paper deals with the "negative Pasteur effect" in yeasts, *i.e.* the inhibition of the alcoholic fermentation under anaerobic conditions and its stimulation in presence of elementary molecular oxygen. This effect was conclusively demonstrated in all *Brettanomyces* strains tested. In three strains of *Br. vini*, including one strain recently reclassified by van der Walt and van Kerken as *Br. intermedius*, mostly no negative Pasteur effect was found in cells harvested from comparatively old cultures, whereas in cells from young cultures a typical effect was obtained.

In addition, a negative Pasteur effect was established in *Torulopsis cylindrica.*

In examining a number of yeast strains at present classified as species of the genera *Torulopsis*, *Saccharomyces* and *Hansenula* no

negative Pasteur effect was observed on calculating the aerobic fermentation without correction for respiration. However, the possibility exists that some of these strains possess a "masked negative Pasteur effect", *i.e.* an effect which may be proven only by applying an appropriate correction for respiration.

All tests were performed on suspensions of resting cells of the yeasts concerned in distilled water.

The taxonomic implications of the results are discussed.

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IN VITRO ACTIVITY OF A COMBINATION OF COLIMYCIN AND TETRACYCLINE AGAINST *PSEUDOMONAS AERUGINOSA* AND *ESCHERICHIA COLI*

by

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Although many investigations have been made into the action of antibiotics in combination, the field of indications for the use of antibiotic combinations has remained limited. With a view to various disadvantages of combined antibiotic treatment, several investigators (*e.g.* BUNN, 1959; LACEY, 1960) have attempted to outline the range of indications with some exactness.

A few combinations have found their definite place in the therapeutic control of certain infectious diseases. Penicillin combined with streptomycin is a generally accepted weapon in cases of enterococcal endocarditis. There seem to be valuable arguments in favour of the combined use of streptomycin and a tetracycline in brucellosis (SHAFFER, KUCERA and SPINK, 1953). *In vitro* demonstration of a synergism between penicillin and kanamycin against *Staphylococcus aureus* and *Proteus* (JADIN, 1960; ANDRIEU, MONNIER and BOURSE, 1959; MOUTON, 1961) facilitated introduction of this combination in the treatment of infections involving these bacteria.

In many cases, *in vitro* experiments also revealed a synergism between polymyxin-B and oxytetracycline against *Pseudomonas aeruginosa* (BUNN, CANARILE and OSBORNE, 1954; VACCA, 1956). The disadvantage of the toxic characteristics of polymyxin-B seemed to be considerably reduced when its dosage could be decreased as a result of its combination with oxytetracycline. The investigation made by HENNEBERG and MÜLLER (1954), who demonstrated a very marked divergence in sensitivity in a single strain of *Pseudomonas aeruginosa*, suggests to us that this synergism is based on suppression, by the oxytetracycline, of the growth of clones more resistant to polymyxin. This view is also taken by LACEY (1960). However, the growth curves presented by BUNN *et al.* (1954) on the basis of their experiments seem to indicate that a true synergism existed. Another publication by BUNN (1959) shows that synergism between polymyxin and tetracycline — which in his opinion makes it possible to reduce the dose of polymyxin to such an extent as to eliminate untoward side-effects – also occurs *in vivo*.

Although the risk of toxic symptoms caused by polymyxin may be reduced by combining it with tetracycline, so that its dosage can be lowered, a less toxic antibiotic of equivalent efficacy nevertheless remains preferable. It seems that, in the past few years, polymyxin is gradually ousted by the related antibiotic colimycin, which is also well active against *Pseudomonas aeruginosa*. Although less effective per unit of weight than polymyxin (MARTIN *et al.*, 1959), colimycin is considerably less toxic despite the higher dosage required. Yet some side-effects have been observed. HALL (1960) reported on a number of patients in whom he observed pain at injection, transient dizziness and paraesthesia. MARTIN *et al.* (1959) saw an allergic reaction. In view of the chemical structure, which is related to that of polymyxin, nephrotoxic manifestations must also be expected, although nephrotoxicity has not so far been demonstrated.

MARTIN *et al.* make mention of a synergism between colimycin and tetracycline against three strains of *E. coli*, *Pseudomonas aeruginosa* and *Shigella sonnei*, respectively. MCCABE and JACKSON (1960) frequently found an antagonism and sometimes a synergism between colimycin and other antibiotics against *E. coli*. Whether tetracycline plays a role in the cases of antagonism is left undiscussed.

We have carried out a number of experiments to determine the extent to which a true synergism exists between colimycin and tetracycline against *Pseudomonas aeruginosa* and *E. coli*, and to establish whether this synergism occurs regularly. Because the results of these experiments in many cases remained inconclusive as to whether synergism or mere addition was involved, growth inhibition curves were obtained. This was also hoped to give some indication of the nature of the synergism (*i.e.* whether this is based on suppression of the growth of resistant clones or is a true synergism).

In so far as it could be justified to reach a conclusion regarding *in vivo* applicability on the basis of *in vitro* experiments, an attempt will be made to form a conclusion as to the possible range of indications for this antibiotic combination.

METHODS.

The bacterial strains included in this investigation were all isolated from material sent to the laboratory to be tested for pathogenic micro-organisms. Nearly all strains originated from infected urine.

Determinations of the minimum bacteriostatic and bactericidal concentrations of colimycin[1]), tetracycline[2]) and these two antibiotics in combination, were carried out with the aid of the serial dilution method, using the technique described in a previous publication (MOUTON, 1961). In these experiments we used meat broth because experience has taught that minimum inhibitory concentrations of antibiotics found with this medium can be used as a basis of dosage and of *in vivo* antibiotic blood concentrations to be attained at this dosage. In the same publication it was explained how a conclusion as to the presence or absence of synergism was reached on the basis of the findings obtained.

The term synergism implies that the joint effect of two antibiotics exceeds the sum of the effects of the two separate antibiotics. The term addition is used when the joint effect equals or almost equals the sum of the separate effects. Whenever the experiments did not definitely reveal whether addition or synergism was involved (see Tables 1 and 2), our preliminary presumption was that addition existed.

In determining synergism or addition on the basis of diffusion of antibiotics in a blood agar plate inoculated with the strain to be tested, we employed ELEK and HILSON's modification (1953) of the replica technique of LEDERBERG and LEDERBERG (1952).

Growth inhibition curves of bacterial cultures in the presence of both antibiotics in varying concentrations and concentration ratios, were obtained as follows. Tubes containing 16, 18 and 20 ml

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[1]) Belcomycine (Rog. Bellon).

[2]) Achromycin (Lederle).

TABLE 1.

Minimum bacteriostatic (Msc) and bactericidal (Mcc) concentrations of colimycin (U/ml), tetracycline (γ/ml) and the combination of both on strains of *Pseudomonas aeruginosa*. Conclusions on synergism with regard to the bacteriostatic (S) and the bactericidal (C) action.

Strain no.	Colimycin		Tetracycline		Colimycin + Tetracycline		Synergism	
	Msc	Mcc	Msc	Mcc	Msc	Mcc	S	C
27	62.5	125	10	20	31.2 + 5	62.5 + 5	—	—
28	62.5	125	10	20	7.8 + 5 15.6 + 2.5	31.2 + 5 15.6 + 10	— +	+ —
C2	62.5	125	10	20	31.2 + 5	62.5 + 5	—	—
C3	62.5	125	20	40	31.2 + 10	62.5 + 5	—	—
C4	62.5	125	10	20	15.6 + 2.5 7.8 + 5	31.2 + 2.5 15.6 + 10	+ —	+ —
C5	62.5	125	20	40	31.2 + 5	31.2 + 10	—	+
C6	62.5	125	10	20	15.6 + 2.5 7.8 + 5	31.2 + 5 7.8 + 10	+ —	+ —
C7	62.5	125	20	80	31.2 + 5	125 + 1.25	—	—

TABLE 2.

Minimum bacteriostatic (Msc) and bactericidal (Mcc) concentrations of colimycin (U/ml), tetracycline (γ/ml) and the combination of both on strains of *E. coli*. Conclusions on synergism with regard to the bacteriostatic (S) and the bactericidal (C) action.

Strain no.	Colimycin		Tetracycline		Colimycin + Tetracycline		Synergism	
	Msc	Mcc	Msc	Mcc	Msc	Mcc	S	C
21	100	100	10	20	6.25 + 5 12.5 + 2.5	6.25 + 5 12.5 + 2.5	— +	+ +
22	100	100	160	320	50 + 10 25 + 40	50 + 10 25 + 40	— +	— +
33	100	100	2.5	10	6.25 + 2.5 12.5 + 1.25	6.25 + 2.5	— —	+
F6	50	100	10	20	12.5 + 2.5 6.25 + 5	6.25 + 5	+ —	+
F7	100	100	5	20	12.5 + 2.5 25 + 1.25	6.25 + 5	— +	+
F8	50	100	5	10	6.25 + 2.5 12.5 + 1.25	6.25 + 2.5	— +	+
F10	200	200	10	40	6.25 + 5 25 + 2.5	6.25 + 25	— +	+
F16	200	200	320	320	50 + 80	100 + 80 50 + 160	+	— —
F17	200	200	160	320	100 + 10 50 + 40	100 + 10 50 + 40	— +	— +

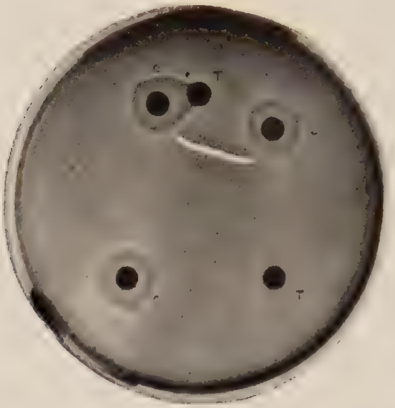
nutrient broth (Difco) (for technical reasons we refrained from using the meat broth we employed in the tube dilution tests) were incubated overnight at 37° C. to ensure an initial temperature of 37° C. in the experiment and also to verify sterility. Quantities of 2 ml (containing ten times the desired antibiotic concentration in nutrient broth) were then added so that each tube finally contained 20 ml antibiotic dilution in nutrient broth. The antibiotic dilutions were freshly prepared every day. The initial bacterial concentration was determined by counting the colonies on plates prepared with nutrient agar (Difco) and a 10^7 dilution of the preliminary culture. One drop (1/30 ml) of this preliminary culture in meat broth was inoculated into all tubes. Incubation was effected under constant rotation at 37° C. Samples of 0.1 ml were obtained after 2, 6 and 24 hours from those tubes in which no growth was discernible; the bacterial concentrations of these samples were determined by the plate count technique. The bacterial concentrations in the tubes in which visible growth had occurred, were determined with the aid of a Vitatron universal colorimeter ($\lambda = 593\,\mathrm{m}\mu$). Standard curves had been plotted for each bacterial type separately. Synergism was accepted when the bactericidal activity of the antibiotic combination was unmistakably more rapid than that of *a*. double the concentration of colimycin used in the combination; *b*. double the concentration of tetracycline used in the combination.

RESULTS.

By means of the tube dilution method it was possible to demonstrate a synergism between colimycin and tetracycline (Table 1) against some strains of *Pseudomonas aeruginosa* (numbers 28, C4, C5 and C6).

In all these cases the synergism seemed dependent on the concentration ratio between the two antibiotics. This, however, must probably be ascribed to the limitations of the method. In the remaining strains it was impossible by this method to determine whether addition existed (as was presumed for the time being) or synergism.

Application of the ELEK and HILSON technique (1953) showed (Fig. 1 and 1a) that subinhibitory concentrations of colimycin, combined with subinhibitory concentrations of tetracycline, can have a bactericidal effect on *Pseudomonas aeruginosa*. This, of course, furnishes no information on synergism as defined under the heading Methods.



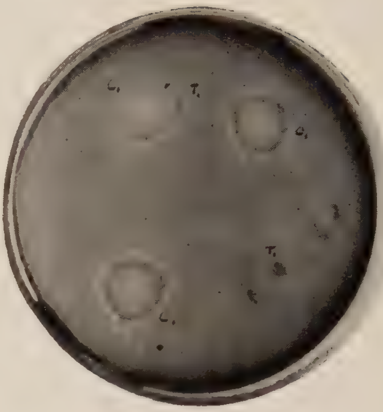


Fig. 1. *Pseudomonas aeruginosa* strain no. 28 on blood agar. Inhibition zones around sensitivity discs containing 250 U colimycin (C) and 35 γ tetracycline (T) respectively. The arrow indicates the area of growth inhibition by subinhibitory concentrations of both antibiotics.

Fig. 1a. Replica plate of plate in Fig. 1. The arrow indicates the area of bactericidal activity by subinhibitory concentrations of both antibiotics.

Colimycin and tetracycline in certain concentration ratios were demonstrably synergistic against all strains of *E. coli* examined. (Table 2).

The question as to whether this synergism is dependent on concentration, and the question as to its mechanism of action, cannot be answered on the basis of these tube dilution tests.

A striking feature of the strains of *Pseudomonas aeruginosa* was that the growth inhibition curves obtained in principle showed the same course in all cases. As examples we selected the curves of strain no. 28 (synergism in tube dilution test – Fig. 2) and those of strain no. C2 (no synergism in tube dilution test – Fig. 3).

We found that growth inhibition by the antibiotic combination was about as rapid as that by double the colimycin concentrations used in the combination. In some cases the curve of the antibiotic combination showed a more favourable course than that of colimycin in double the concentration used in the combination (*e.g.* strain no. C2, Fig. 3); other strains showed the reverse (*e.g.* strain no. 28, Fig. 2). In the case of strain no. 28, the course of the curves initially indicates synergism, and subsequently addition. In strain no. C2, mild synergism can be accepted. If tetracycline is added to bactericidal concentrations of colimycin, then the bactericidal action is

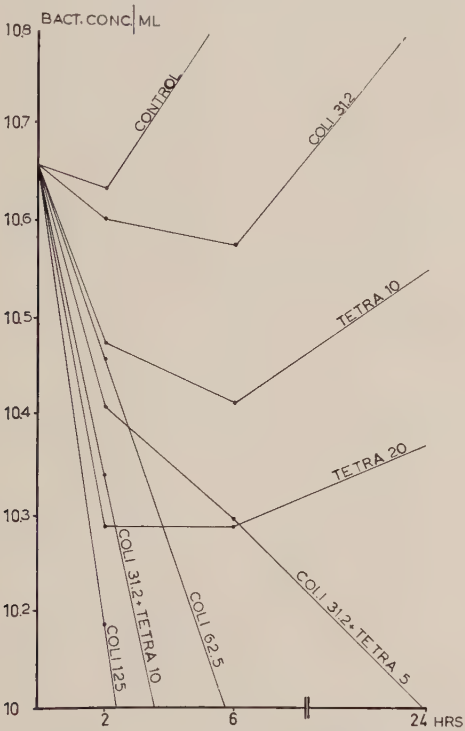


Fig. 2. Growth inhibition curves of *Pseudomonas* strain no. 28 in the presence of colimycin, tetracycline and the combination of both. The numbers indicate the concentrations of the antibiotics per ml.

even more rapid (Fig. 3). The nature of the interaction between the two antibiotics in this respect is not easily determined. It is beyond doubt, however, that at least an additive effect is involved even at high concentrations.

Among 35 strains of *Pseudomonas aeruginosa* examined, we found none that was only inhibited by very high concentrations of tetracycline (see *E. coli* strains no. 22, F16 and F17). It was impossible to establish, therefore, whether the two antibiotics still have an additive effect at a very high minimum inhibitory concentration of tetracycline.

The growth inhibition curves of *E. coli* in the presence of colimycin, tetracycline or their combination show a partly different pattern. A typical example is shown in Fig. 4.

During the first few hours, the course of the curves at best indicates addition. The course of the lines of colimycin 31.2 U/ml

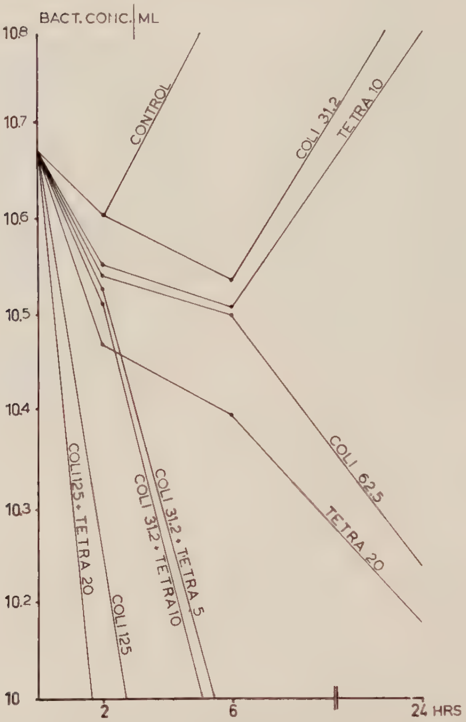


Fig. 3. Growth inhibition curves of *Pseudomonas* strain no. C2 in the presence of colimycin, tetracycline and the combination of both. The numbers indicate the concentrations of the antibiotics per ml.

and 62.5 U/ml points in the direction of a culture of bacteria showing divergent sensitivity to colimycin. The synergism revealed by the dilution tests is largely based, therefore, on suppression of the growth of the more resistant bacteria in the culture, and partly on an additive effect. Fig. 5 shows that the same holds true for an *E. coli* strain (F10) which is less sensitive to colimycin. In so far as this can be established by a test of this kind with a highly bactericidal antibiotic such as colimycin, the figures indicate that the nature of the interaction between the antibiotics is not significantly influenced by changes in the antibiotic concentrations.

The growth inhibition curves obtained for a few other strains of *E. coli* (no. 33 and F6) showed that addition of tetracycline to highly bactericidal concentrations of colimycin did not essentially alter the nature of the interaction.

Fig. 6 shows that, even in the case of tetracycline-resistant strains

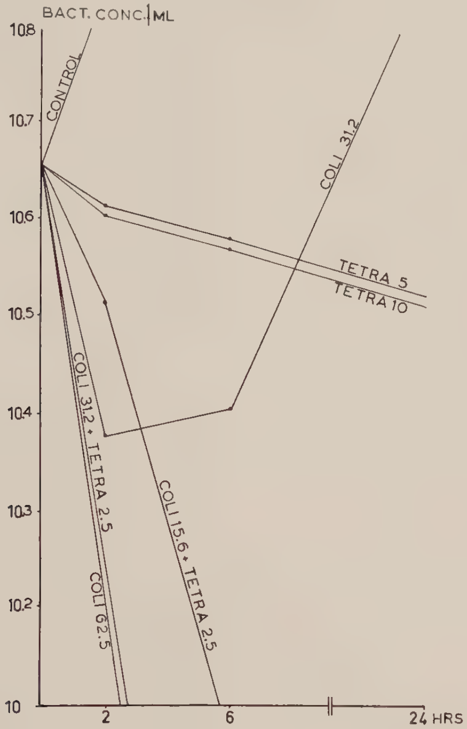
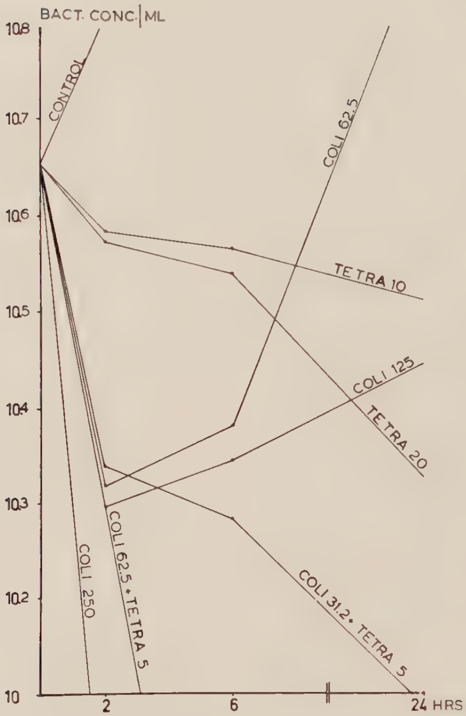


Fig. 4. Growth inhibition curves of *E.coli* strain no. F7 in the presence of colimycin, tetracycline and the combination of both. The numbers indicate the concentrations of the antibiotics per ml.

of *E. coli*, suppression of the growth of micro-organisms more resistant to colimycin can be caused by tetracycline in a concentration far below the minimum inhibitory concentration of this antibiotic.

DISCUSSION.

Qualitatively, the results of the tube dilution tests virtually agree with those of the growth inhibition experiments. Slight differences, however, can be observed in some cases. In the case of strain no. C2, for example, the tube dilution tests showed only addition, whereas the growth inhibition experiments indicated slight synergism. This minute discrepancy must probably be ascribed to the fact that – in the tube dilution method – the choice of the (twofold) concentrations



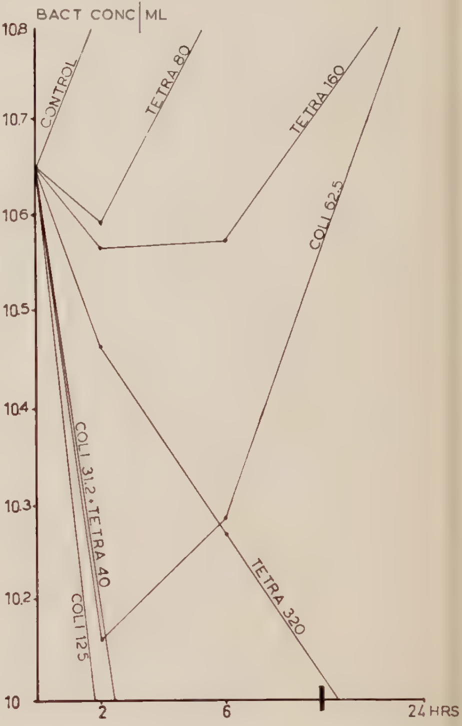


Fig. 5. Growth inhibition curves of *E.coli* strain no. F10 in the presence of colimycin, tetracycline and the combination of both. The numbers indicate the concentrations of the antibiotics per ml.

Fig. 6. Growth inhibition curves of *E.coli* strain no. F16 in the presence of colimycin, tetracycline and the combination of both. The numbers indicate the concentrations of the antibiotics per ml.

may exert some influence on the result. In view of the results of preliminary tube dilution tests with nutrient broth, the differences between the two test series in absolute values of minimum inhibitory concentrations of the antibiotics must be ascribed to the use of different media. This observation emphasizes that inhibitory antibiotic concentrations found *in vitro* are dependent on the method employed, and cannot always be applied to *in vivo* conditions. It is none the less true that the nature of the interaction between two antibiotics is not influenced by this; only the absolute values found are different.

If a conclusion is to be formed regarding the nature of the interaction between colimycin and tetracycline against *Pseudomonas aeruginosa* and *E. coli*, it should be the following.

Both the tube dilution tests and the growth inhibition curves warrant the conclusion that the two antibiotics have an additive effect on *Pseudomonas aeruginosa*. In some cases a synergism – albeit only slight – is undoubtedly involved. These findings, jointly with those obtained by replica plate experiments, show that subinhibitory concentrations of colimycin are capable of a bactericidal action in the presence of subinhibitory concentrations of tetracycline.

The interaction of the two antibiotics against *E. coli* is virtually equivalent to that against *Pseudomonas* strains, but a second factor is of great importance, *viz.*, suppression, by tetracycline, of the growth of clones more resistant to colimycin. The result of these two mechanisms is an unmistakable synergism in the wider sense of the term.

The values of the separate minimum inhibitory antibiotic concentrations have no distinct effect on the nature of the interaction between the two antibiotics. Within the limitations of our methods we found no indication that this interaction is dependent on the concentrations of each of the antibiotics.

Although it has been pointed out that purely quantitative application of these data to *in vivo* conditions is unjustifiable, there seems to be no objection to doing this qualitatively. In some cases in which the resistance pattern makes it necessary to resort to colimycin (frequently the case in infections with *Pseudomonas aeruginosa*) although its possible side-effects might ultimately exert an untoward influence it seems worthwhile to start trials with small doses of colimycin combined with tetracycline. In our opinion, combined colimycin and tetracycline medication can also be tried in cases of infection caused by bacteria of moderate sensitivity to colimycin, in which other antibiotics are contraindicated for any reason. In the final analysis, however, the value of simultaneous administration of the two antibiotics will have to be determined by comparative clinical investigation.

Summary.

The *in vitro* activity of a combination of colimycin and tetracycline against *Pseudomonas aeruginosa* and *Escherichia coli* was

studied with the aid of tube dilution tests, Elek and Hilson's modification of the replica technique, and growth inhibition curves. The interaction between the two antibiotics was as a rule of an additive nature, but a slight synergism was observed in some instances. The values of the minimum inhibitory concentrations did not seem to influence the nature of the interaction. A considerable synergism observed in all tube dilution tests with *E. coli* strains is attributed partly to addition and partly to suppression of the growth of more colimycin-resistant bacteria by the tetracycline.

Possible *in vivo* applications of this antibiotic combination are briefly discussed.

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OBSERVATIONS ON THE MORPHOLOGY OF *THIOVULUM MAJUS* HINZE[1]

by

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A. L. HOUWINK

(Received September 25, 1961).

Introduction.

Thiovulum majus Hinze is at present considered to be a colorless sulfur bacterium and is classified as a member of the *Thiobacteriaceae* in the 7th Edition of Bergey's Manual (Breed *et al.*, 1957). It is generally found in marine environments where O_2 meets H_2S formed from decaying vegetable or animal matter. The cells are strongly chemotactic and concentrate in characteristic patterns in regions of optimal O_2 and H_2S concentrations. Generally the cells are almost entirely filled with sulphur inclusions which disappear when the H_2S concentration in the medium decreases. A more detailed survey of the characteristics of *Thiovulum* and the methods for its cultivation is given by la Rivière (1962).

The morphology of *Thiovulum* presents some striking features: the cells are spherical to ovoid, the longer diameter ranging in length from 5 – 25 μ; they divide by longitudinal fission and are highly motile, revolving round the longer axis and proceeding in the direction of the latter. So far, only Hinze (1913) has claimed demonstration of flagella; by staining fixed specimens with fuchsin he found these to be peritrichously arranged. However, subsequent workers failed to corroborate this result and as a consequence the mechanism of locomotion of *Thiovulum* is still considered an unsolved problem.

While the large size and the longitudinal manner of division of *Thiovulum* together provide sufficient reason to question the desirability of classing this organism with the bacteria, the inner structure of the cell has also been shown to deviate in several respects from that normally encountered in bacteria. The exemplary cytological investigations of Fauré-Fremiet and Rouiller (1958) have demonstrated the following features: (1) an extensive system of intracellular membranes; (2) a so called "organite antapical", a disc-shaped organelle situated at the inner surface of the cell envelope and containing about a hundred fibrils oriented perpendicularly to the cell surface, each with a length of 2500–3500 Å and a width of circa 100 Å; (3) a ribbon shaped organelle of Feulgen-positive material near or at the cell surface.

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[1] Part of the results have been briefly communicated at the Symposium on Marine Microbiology held at Chicago in April, 1961 (la Rivière, 1962).

Because they found no flagella, Fauré-Fremiet and Rouiller were forced to consider the possibility that in *Thiovulum* the mechanism of locomotion is also of an unusual type, perhaps involving the organite antapical.

The complex of structural elements found by Fauré-Fremiet and Rouiller points to a degree of differentiation as yet quite exceptional for a bacterium and makes *Thiovulum* a promising object for further comparative cytological studies (Murray, 1960). We started our investigation by re-examining the locomotion problem because of its primary importance for morphological characterisation.

MATERIALS AND METHODS.

The cells were harvested from enrichment cultures; contaminants which might interfere were removed by repeated chemotactic migration through sterile sea water (la Rivière, 1962). The diameters of the cells usually ranged between 5 and 10 μ. In some populations the diameters of the cells were larger and ranged between 20 and 25 μ.

For fixation, washing and embedding in agar a stock solution (solution S) was used which, except for a slight modification, was prepared according to Ryter *et al.* (1958):

Acetate-veronal buffer:		
19.428 g Na-acetate 29.428 g Na-veronate 34 g NaCl	in 1 l water	5 ml
0.1 N HCl		3 ml
Sea water		17 ml
1 M $MgCl_2$		0.25 ml

The final pH was 7.9.

For fixation of cells to be sectioned 1% OsO_4 and 0.005% peptone were added to solution S which then was mixed with an equal volume of a cell suspension in sea water. After 16 hrs the cells were washed in solution S and embedded in solution S with 2% agar. Agar blocks of 0.5 mm^3 were suspended for 1 hr in a solution containing 0.5% uranylacetate, 0.1% ammonium acetate and 0.25% EDTA in distilled water adjusted to a pH of 7.9 with 1 N NaOH (E. F. J. van Bruggen, personal communication). Dehydration was performed in acetone-water mixtures and Vestopal W was used for final embedding.

For the examination of intact cells solution S with 1.5% OsO_4 and 0.005% peptone was used as a fixative. After fixation the cells were washed in distilled water. Instead of being centrifuged, they were allowed to sink to the bottom. After drying they were shadowed with platinum.

RESULTS AND DISCUSSION.

Shadowed specimens did not reveal flagella if the cells were not fixed in suspension but just washed and dried; nor did they show any if the cells were killed by dessiccation or by exposing them to a temperature of 37° C. for 15 min. prior to fixation. However, if the fixative was added directly to a suspension of actively moving cells, flagella were observed to radiate from the greater part of the circumference of the cells (Fig. 1). Small (5–10 μ) and big (20–25 μ) cells proved to have the same arrangement of flagella. The width of the flagella was about 100 Å, as is usual with bacteria. The electron micrographs strikingly resemble Hinze's drawing (1913), reproduced in Fig. 2.

It appears therefore that the flagella become detached from the cells if the latter are killed before fixation. This may explain why Hinze's observations failed to be corroborated by later investigators.

Besides flagella, some cells had a "tail" presumably of a slimy character (Fig. 3). This may explain our light-microscopical observations of *Thiovulum* cells sometimes dragging along small particles of debris, apparently sticking to an – invisible – slimy tail, 1–8 times as long as the organism.

The picture of a *Thiovulum* cell as shown by our sections is the same as that which Fauré-Fremiet and Rouiller obtained from

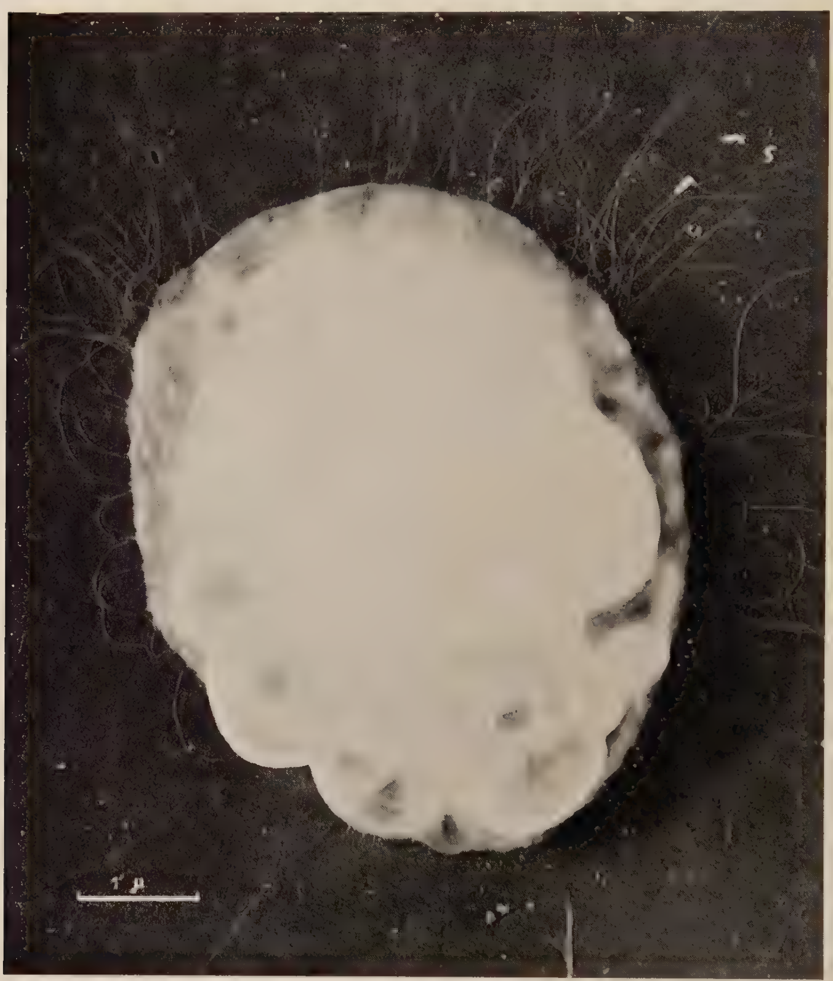

Fig. 1. Electron micrograph of *Thiovulum majus* showing flagella.

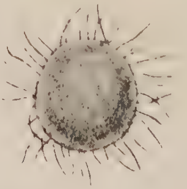

Fig. 2. Hinze's drawing (1913) of a stained specimen of *Thiovulum majus*.

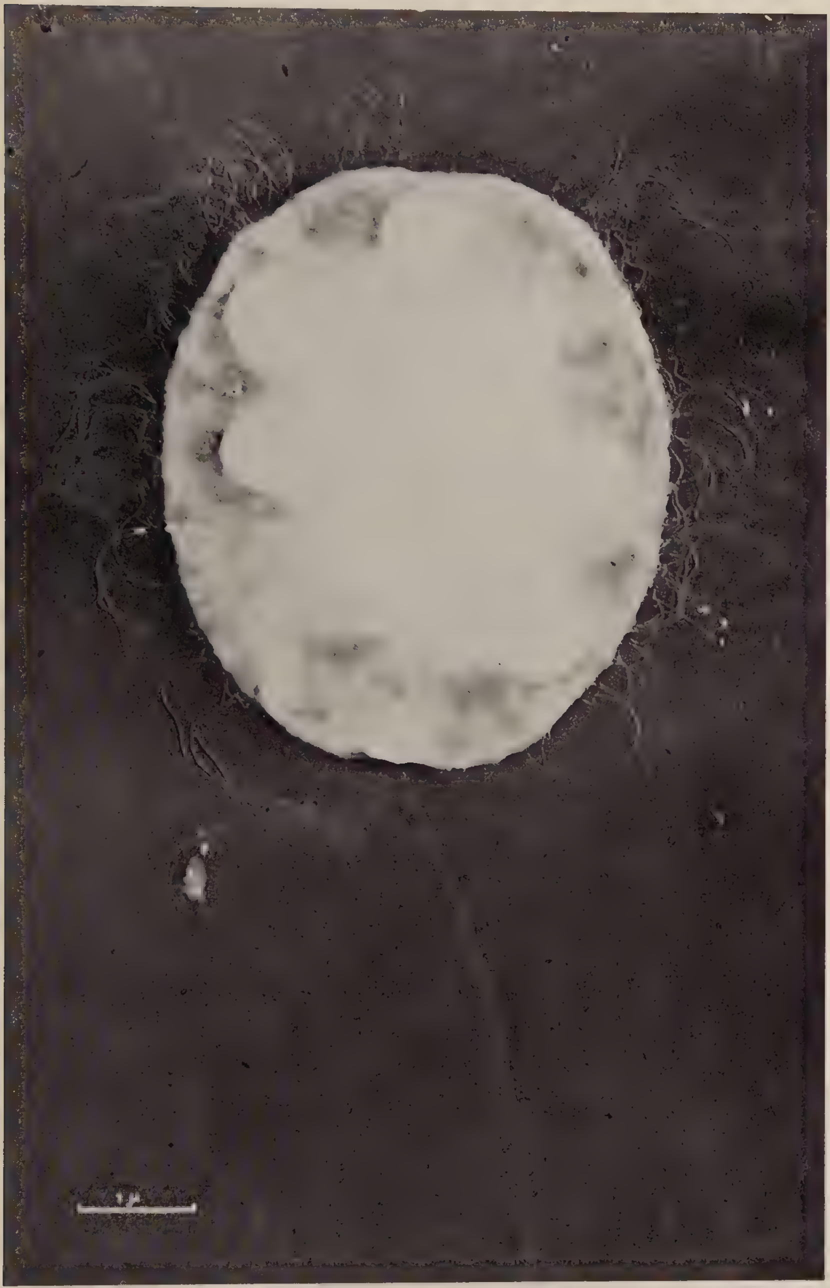

Fig. 3. Electron micrograph of *Thiovulum majus* showing flagella and "tail" of slime.

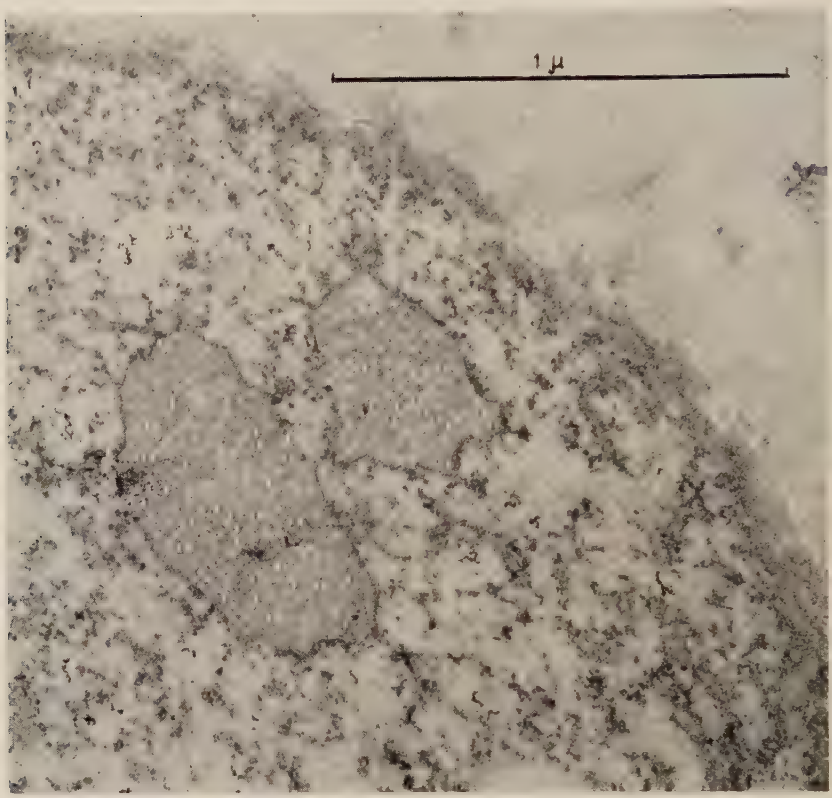

Fig. 4. Section of *Thiovulum majus*. Two fibrillar organelles in close proximity have been sectioned perpendicularly to the fibrils.

theirs. This applies in particular to the "organite antapical" and to the fibrils which seem to be its major components. In some cells we found two of these organelles instead of one (Fig. 4). In every one of these cases, however, the two "organites antapicals" were as near to one another as they would be expected to be if produced by division of a single one. Division of the "organite antapical" precedes cell division. In fact, fission was observed to begin between the twin organelles (Fig. 5).

According to Fauré-Fremiet and Rouiller, stained specimens show that the fibrillar organelle is situated at one pole of the organism, the greater part of the sulfur inclusions being located at the other one. Observations on motile cells led these authors to the conclusion that the pole with the sulfur inclusions is the apex *i.e.*

the foremost part of the cell when the organism is moving. Therefore they named the fibrillar organelle – situated at the opposite pole – "organite antapical".

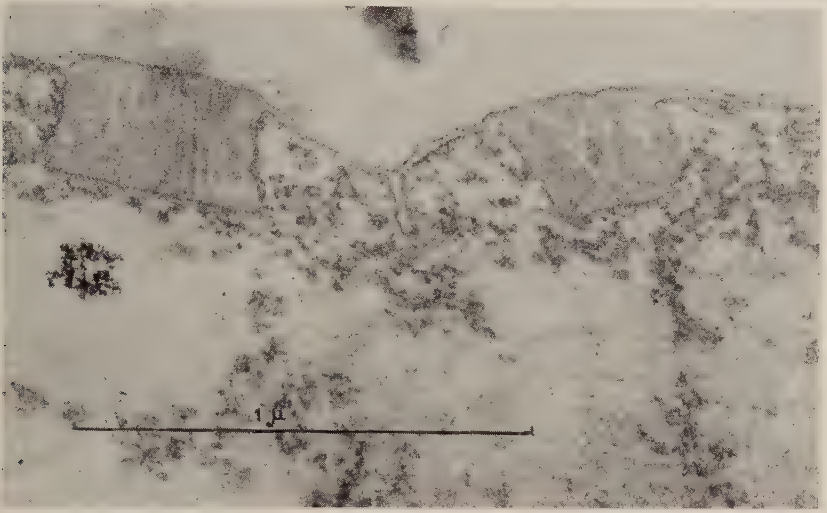

Fig. 5. Section of *Thiovulum majus* showing location of fibrillar organelles between inner and outer membranes and the beginning of cellular fission between twin organelles, sectioned parallel to fibrils.

Our observations on motile cells of various populations do not corroborate those of Fauré-Fremiet and Rouiller on this point. It is our experience that one may find sulfur inclusions at the antapical pole as well as at the apical one, or at both poles at the same time. Therefore it does not seem certain that the fibrillar organelle is always at the rear end of the organism, so "organite antapical" may not be a fit name for it. We want to emphasize, however, that we have never seen a cell reverse its poles with respect to its direction of motion; both ends are therefore not equivalent and consequently the cell must also possess a definite polarity in the physiological sense.

Since flagella have been demonstrated there is no reason left to consider the fibrillar organelle responsible for the motility of the organism, which makes the mystery of its function all the more puzzling.

The outer membrane of the organism is a strikingly thin membrane, circa 50 Å thick. Micrographs not reproduced here show that this membrane is of the common "double membrane" type. Another membrane of similar appearance borders the cytoplasm. The outer membrane meets the outer face of the fibrillar organelle whereas the inner one seems continuous with the membrane at the inner base of the latter (Fig. 5). So one might infer that the organelle is situated between these two membranes, and that the membranes are less closely connected than one might expect if the outer membrane would be the cell wall and the inner one the cytoplasmic membrane. Also, the pictures of cells about to divide show that the outer membrane grows into the fissure between the two daughter cytoplasts, following the inner membrane, but not at a close distance (Fig. 6).

In bacteria, a cell wall as thin as the outer membrane of *Thiovulum* has only rarely been described. An outer membrane of comparable thinness has been found by Bush and Chapman (1961) in a symbiotic bacterium occurring intercellularly in *Periplaneta americana*. Common to both cases may be the absence of osmotic pressure differences at the cytoplasmic membrane; a bacterium in sea water may be able to do without a mechanically strong cell wall and particularly a spherical shape should be easy to maintain. The lack of rigidity of the outer membrane of *Thiovulum* manifests itself if cells are suspended in a half saturated solution of saccharose: both the outer and the inner membranes follow the shrinking cytoplasm, and the shape of the cell – no longer spherical – is now determined by the agglomerated sulfur inclusions; all the same the organism keeps moving.

Like Fauré-Fremiet and Rouiller we observed intracellular membranes. However, the results of our study do not, so far, justify any speculation as to their anatomy and function.

Though the cytoplasm encloses the sulfur inclusions, the latter are not found in our sections since the sulfur has been dissolved in the course of dehydration. Thus the former sulfur inclusions are represented in the sections by electron optically transparent areas. These areas are sharply delineated though not by a membrane, at least not by the common type of double membrane (Fig. 6).

We also found electron optically translucent areas of another kind, larger than the former, not sharply delineated, irregularly shaped and containing indistinct, thin threads (Fig. 6). Similar areas have been found in various bacteria by means of Ryter's technique. At present these bacterial areas are considered to re-

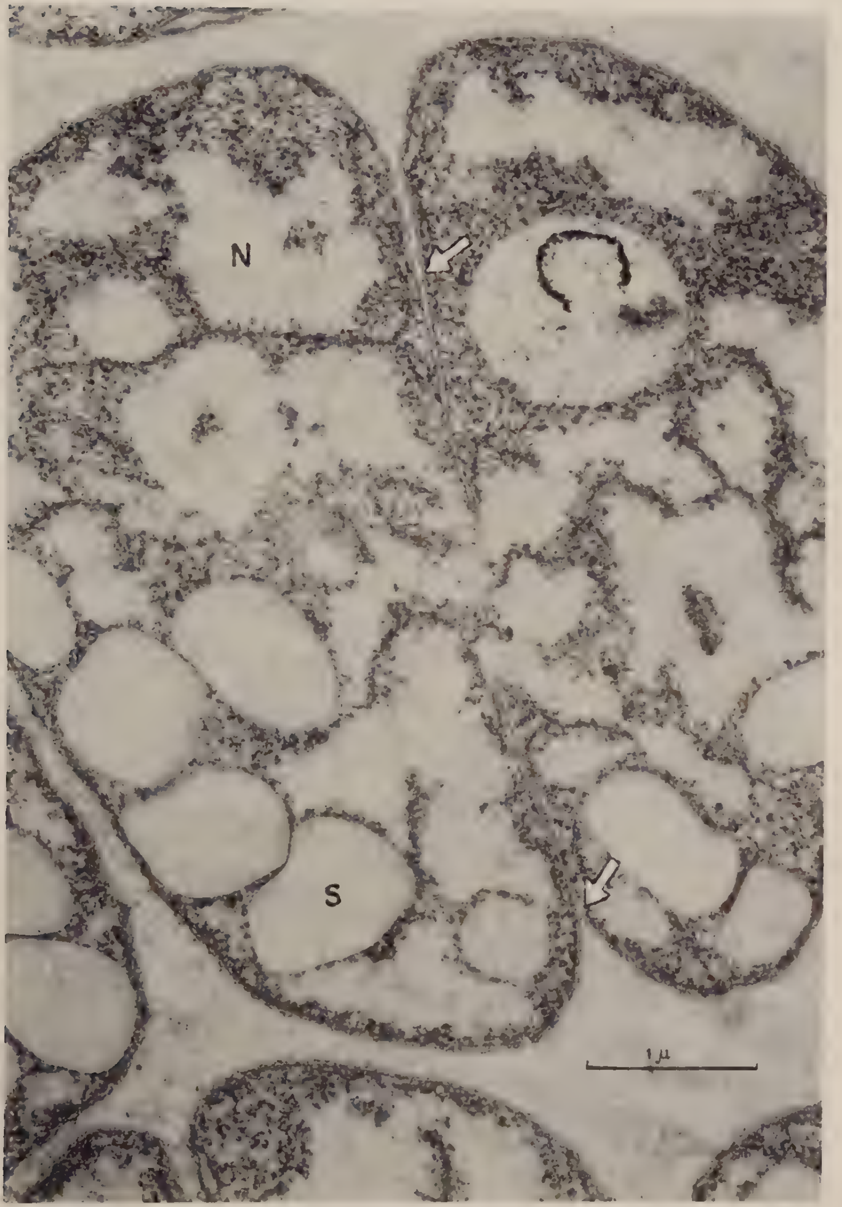

Fig. 6. Section of dividing cell of *Thiovulum majus* showing: (1) retarded growth of outer membrane as compared to that of inner membrane, the arrows indicating end-points of growth of outer membrane within the fissure between the cells; (2) inclusions (S) originally filled with sulphur; (3) areas (N) presumably containing nuclear material.

present the nuclear material of the cell (*cf.* MURRAY, 1960). The same situation may exist in *Thiovulum*.

In conclusion we may state that *Thiovulum* is less different from the bacteria than earlier observations allowed to believe: the organism possesses bacterial flagella and in addition the organisation of its nuclear material may well be identical with that of bacteria.

Summary.

Recent investigations have demonstrated that *Thiovulum majus* Hinze possesses a high degree of cytological differentiation. The nature of the mechanism of its locomotion, however, remained obscure. The results of the present study confirm that the cytology of *Thiovulum* is more highly differentiated than is common in bacteria, but show on the other hand that locomotion is effected by flagella, quite comparable to those of bacteria. Furthermore, evidence is presented suggesting that the organisation of nuclear material in *Thiovulum* resembles that found in bacteria.

Acknowledgement.

The authors gratefully acknowledge the skilled assistance of Miss H. BRAKMAN who prepared the ultra-thin sections.

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(Department of Biology, University of Tokyo, Meguro, Tokyo, Japan).

OBSERVATION OF NUCLEAR FUSION IN LIVING CELLS OF *BACILLUS MEGATERIUM*[1]

by

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(Received May 17, 1961).

Since BADIAN (1930, 1933) reported fusion of nuclear bodies in *Myxococcus virescens*, many other investigators have reported nuclear fusion in Mycobacteria (LINDEGREN and MELLON, 1932); *Bacillus megaterium* (BADIAN, 1935; DELAMATER and HUNTER, 1952); *Bacillus cereus* (PULVERTAFT and HAYNES, 1951); *Escherichia coli* (LEDERBERG, 1956; SCHAECHTER and LAING, 1961); *Aerobacter aerogenes, Myxococcus* and *Chondrococcus* (BISSET, GRACE and MORRIS, 1951); *Actinomyces* (KLIENEBERGER-NOBEL, 1947a); Myxococci (KLIENEBERGER-NOBEL, 1947b); and *Sporocytophaga myxococcoides* (GRACE, 1951; BISSET, GRACE and MORRIS, 1951).

In the present study, cytological evidence has been obtained by means of phase contrast microscopy which indicates the occurrence of nuclear fusion in *Bacillus megaterium.*

MATERIAL AND METHODS.

Bacillus megaterium was obtained from the Culture Service, Terre Haute, Indiana. For the observation of nuclear fusion, cells which had been grown on nutrient agar for twenty hours were transferred to nutrient agar with sodium monofluoroacetate (0.005 M) (KALNITSKY and BARRON, 1947) and $MgSO_4$ (0.003 M). After incubation on this medium for three hours at 30° C., the cells were mounted in liquid paraffin on a microscope slide and covered with a cover glass.

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[1] A part of this work was published in the Japanese Journal of Genetics **34**, 88, 1959 (in Japanese).

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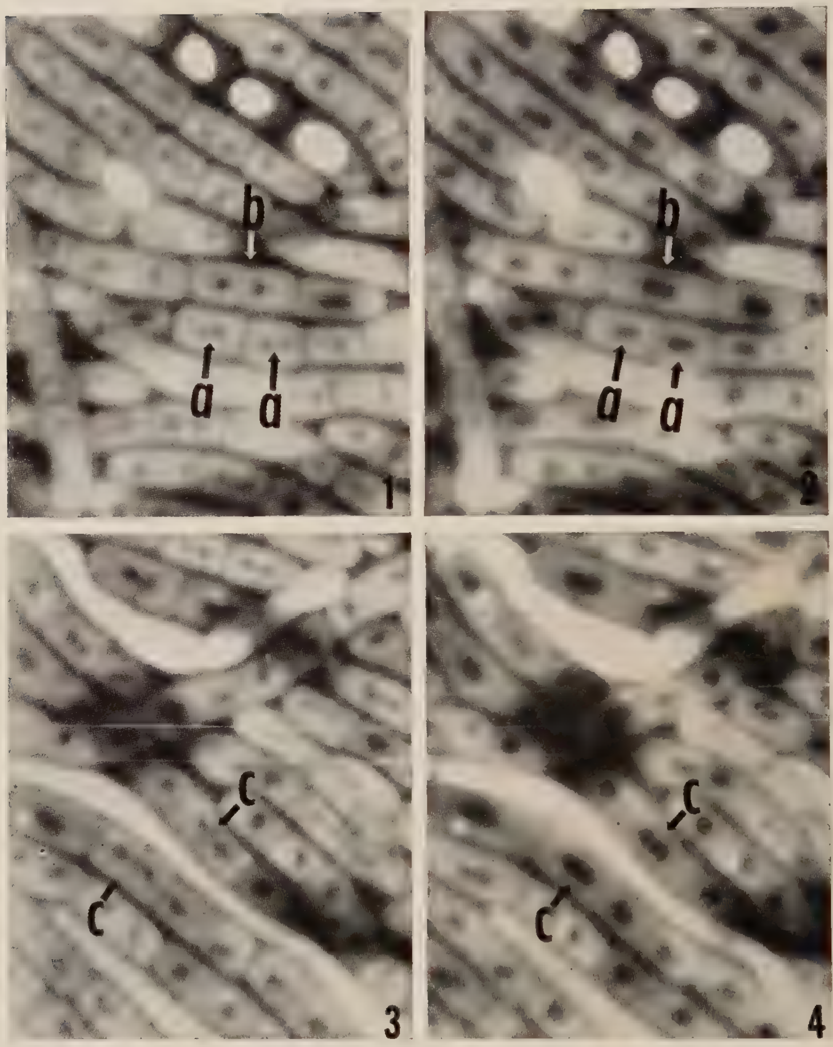

Figures 1-4. These figures constitute two sets of phase contrast micrographs of *Bacillus megaterium* showing nuclear fusion. Figures 2 and 4 were photographed twenty minutes after figures 1 and 3 respectively, and show the occurrence of nuclear fusion during this period. For explanation of details see text.

Observations were carried out with a Tiyoda bright high phase contrast objective (90×).

A Canon 4-SB 35 mm camera and Fuji minicopy film were used to record selected fields at appropriate time intervals.

RESULTS AND DISCUSSION.

Nuclear division has been observed by many investigators using nuclear staining techniques, and can be clearly seen in living, dividing bacteria by phase contrast microscopy (MASON and POWELSON, 1956). Most recently, nuclear fusion under the influence of chloramphenicol has been observed in *E. coli* with the phase contrast microscope (SCHAECHTER and LAING, 1961).

Nuclear fusion is known to occur in bacteria as a result of certain alterations to the environment (KELLENBERGER, 1960; WHITFIELD and MURRAY, 1956). In the present case, the addition of sodium monofluoroacetate to the nutrient agar is considered to bring about the fusion of nuclei in *Bacillus megaterium.*

The nuclear fusion process may be described as follows: after the nuclear division is completed, there are two separate chromatin bodies contained in the cells (Figure 1-a). Those chromatin bodies will become resting nuclear bodies in about twenty minutes (Figure 2-a). The resting nuclear bodies in adjoining cells (Figures 1-b, 3-c) are fused in about twenty minutes (Figures 2-b, 4-c). The nuclear fusion is perhaps carried out in the stage of the resting nucleus.

The author believes that this observation of nuclear fusion in living cells provides valuable confirmatory evidence of the nuclear fusion reported by workers who used stained preparations.

Summary.

Fusion of nuclear bodies was observed by phase contrast microscopy in living cells of *Bacillus megaterium* grown on nutrient agar with added sodium monofluoroacetate. The nuclear fusion is perhaps carried out in the stage of the resting nucleus. The resting nuclear bodies in adjoining cells are fused in about twenty minutes.

Acknowledgement.

The author wishes to thank Dr. T. AKIBA and Dr. T. IINO for their helpful suggestions and Dr. A. YUASA for the use of facilities in his laboratory. The author is also greatly obliged to Dr. GEORGE B. CHAPMAN for the revision of the manuscript.

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(From the National Institute of Public Health, Utrecht, the Netherlands).

INCIDENCE OF RESISTANCE TO CHLORAMPHENICOL AND TETRACYCLINES AMONG 11981 *SALMONELLA* STRAINS ISOLATED IN 1960

by

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(Received September 12, 1961).

In our previous publication (1961) we communicated the results of an inquiry into the incidence of resistance among 12014 *Salmonella* strains examined in the years 1958 and 1959. We found that only 250 of these strains, or 2.08%, were resistant to tetracycline, to chloramphenicol, or to both of these antibiotics. In the same paper we called attention to the desirability to continue the investigation as the use of antibiotics might be expected to increase and this would possibly lead to a more frequent occurrence of resistance.

An important application of the tetracyclines is their use in cattle-feeds. In the Netherlands the addition of these substances to feeds was until recently (September 1959) severely restricted by law; since then many of these restrictions have been abolished, so that a much wider application has become possible.

As was pointed out in our previous publication, the use of chloramphenicol and tetracycline in medical and veterinary practice and also in the preparation of modern cattle-feeds requires that special attention is paid to the development of resistance in the various types of the genus *Salmonella*. In the present investigation the incidence of resistance among *Salmonella* strains isolated in 1960 was ascertained.

In this year 11025 strains were collected in the Netherlands. Of these 5955 were isolated from man, 2034 from animals, and the rest from other sources. In addition we tested 956 strains that had been

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TABLE 1.

Type and frequency of resistance among *Salmonella* strains isolated in the Netherlands in 1960.

Salmonella-types	Man				Animals				Other materials				Total number of strains examined	Total number of resistant strains
	Number of strains examined	Resistant to			Number of strains examined	Resistant to			Number of strains examined	Resistant to				
		T	C	T+C		T	C	T+C		T	C	T+C		
S. anatum	65	2	—	—	44	1	—	—	112	—	—	—	221	3
S. bareilly	135	—	—	—	53	1	—	—	82	—	—	—	270	1
S. blockley	138	1	—	—	22	—	—	—	33	—	—	—	193	1
S. bredeney	403	5	—	—	13	—	—	—	392	5	—	—	808	10
S. derby	4	—	—	—	1	—	—	—	8	1	—	—	13	1
S. dublin	57	1	—	—	1234	7	—	1	22	—	—	—	1313	9
S. give	96	—	—	—	11	1	—	—	170	2	—	—	277	3
S. heidelberg	399	3	2	4	13	—	—	—	114	2	—	—	526	11
S. infantis	49	1	—	2	1	—	—	—	57	—	—	—	107	3
S. java	72	2	—	—	1	—	—	—	18	—	—	—	91	2
S. livingstone	12	—	—	—	3	—	—	—	112	—	1	—	127	1
S. manchester	119	—	1	—	2	—	—	—	55	—	—	—	176	1
S. newport	171	4	—	—	16	—	—	—	44	1	—	1	231	6
S. oranienburg	22	—	—	3	5	—	—	—	28	—	—	—	55	3
S. paratyphi B	253	—	1	1	—	—	—	—	62	—	—	—	315	2
S. taksony	7	—	—	—	3	—	—	—	73	1	—	—	83	1
S. typhimurium	3253	37	9	5	365	14	—	2	610	8	1	—	4228	76
S. washington	81	2	1	—	18	—	—	—	339	1	—	—	438	4
Salmonella group B	21	5	—	—	1	—	—	—	0	—	—	—	22	5
Other types	598	—	—	—	228	—	—	—	705	—	—	—	1531	—
Totals resistant		63	14	15		24	—	3		21	2	1		143
Total general	5955				2034				3036				11025	

Abbreviations: T = tetracycline; C = chloramphenicol.

sent to us from the Dutch overseas territories, more especially from the Dutch Antilles.

As in our previous investigation, the resistant strains were very carefully screened. For this purpose a quantitative method was used: each strain was inoculated on an agar-slant containing 50 μg/ml chloramphenicol and on another containing 25 μg/ml tetracycline. These amounts roughly correspond to 10 times the normal values of the minimum inhibitory doses of these drugs *in vitro*. These values we have found to differ but little for the various types of the genus *Salmonella*. In order to exclude any possibility of a contamination with resistant bacteria, such as *Proteus* or *Pseudomonas*, every strain which proved to be able to grow on one or both of the slants was typed again. The degree of resistance of the *Salmonella* strains screened was then determined by preparing a diluted suspension of each strain in saline and inoculating this on a number of slants of 5% sheep blood agar (pH 7.2 to 7.4) containing different amounts of the antibiotics. The concentration used were: 0–5–10–25–50–100 μg of tetracycline (or chloramphenicol) per ml. A strain was only reckoned to be resistant, if the minimum inhibitory concentration thus determined showed that the strain in question was at least 10 times less sensitive than normal.

The results of the investigation are given in the tables 1 and 2.

TABLE 2.

Type and frequency of resistance among *Salmonella* strains isolated in the Netherlands Antilles in 1960.

Salmonella types	Total number of strains examined	Resistant to			Total number of resistant strains
		T	C	T + C	
S. chester	13	5	—	—	5
S. havana	24	2	—	—	2
S. montevideo	53	—	—	1	1
S. oranienburg	398	8	3	—	11
S. panama	112	1	—	4	5
S. san diego	45	1	—	—	1
S. typhimurium	41	5	—	—	5
other types	270	1	—	—	1
Totals	956	23	3	5	31

As these tables show, out of the 11025 *Salmonella* strains found in the Netherlands 142, or 1.29%, were resistant to tetracycline or chloramphenicol or to both of these drugs. Of the 956 strains isolated in the Netherlands Antilles 31, or about 3.2%, were resistant. The more frequent occurrence of resistance among the latter strains is probably due to the fact that broad spectrum antibiotics are more freely used for therapeutic purposes in these territories. Whereas in the years 1958 and 1959 the frequency of resistance in the Netherlands was 2.08%, it dropped to 1.29% in 1960. This decrease set in notwithstanding the greatly increased use of tetracycline in cattle-feeds in that year. We believe that this fact corroborates the surmise that we put forward in our previous publication *i.e.* that the resistance of *Salmonella* to antibiotics is practically entirely due to the use of these drugs for therapeutic purposes.

Summary.

The incidence of resistance to tetracycline and chloramphenicol among *Salmonella* strains, which had been 2.08% in the years 1958 and 1959, dropped to 1.29% in 1960, in spite of the greatly increased use of tetracyclines in cattle-feeds in that year. It is therefore suggested that the resistance to antibiotics found among *Salmonella* strains is practically entirely due to the therapeutic use of these drugs.

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(From the National Institute of Public Health, Utrecht, the Netherlands).

THREE NEW *SALMONELLA* TYPES (*S. BILTHOVEN, S. WASSENAAR* AND *S. BONAIRE*)

by

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and **H. M. C. C. WILLEMS**[3]

(Received May 10, 1961).

S. bilthoven was isolated from the organs of a chameleon (*Chamaeleo vulgaris*); *S. wassenaar* from a lizzard (*Amphibolurus barbatus*).

These animals all belonged to zoos in the Netherlands and these two types were isolated by P. ZWART and F. G. POELMA[4]).

S. bonaire was isolated from the faeces of a cow on the Island Bonaire by Dr. J. G. A. BORGHANS, Public Health Laboratory, Willemstad, Curaçao.

In table 1 the biochemical data of the three new types are shown. All strains liquefied gelatin within two days when tested according to the technique of LAUTROP.

S. wassenaar and *S. bonaire* were both dulcitol negative, salicine positive and KCN positive.

In tables 2 and 3 the serological data are given for the three new *Salmonella* types.

S. bilthoven has the antigenic formula 47 ac : a : —.

After absorption of O-serum *S. wassenaar* by *S. greenside* a rest titer of 1 : 160 remained. This absorbed serum gave no agglutination with all known *Salmonella-* and *Arizona*-antigens, except with *S. bonaire* 1 : 80.

After absorption of O-serum *S. bonaire* by *S. greenside* a rest titer of 1 : 160 remained. This absorbed serum gave no agglutination

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[3]) Technical assistant, National Salmonella Centre.

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TABLE 1.

Biochemical behaviour of *S. bilthoven*, *S. wassenaar* and *S. bonaire*.

	glucose + gas	mannitol	adonitol	dulcitol	inositol	lactose	salicine	sucrose	indol	H_2S (T.S.I.)	ammoniumcitrate	methylred	Voges-Proskauer	urea	phenyl-alanine	malonate	KCN	arabinose	rhamnose	trehalose	xylose	glycerol (Stern)	d-tartrate	l-tartrate	i-tartrate	citrate	mucate	gelatin
S. bilthoven	+ +	+	—	+	—	—	—	—	—	+	+	+	—	—	—	+	—	+	+	+	+	+	+	+	—	+	+	+
S. wassenaar	+ +	+	—	—	—	—	+	—	—	+	+	+	—	—	—	—	+	+	+	+	+	—	+	+	—	+	+	+
S. bonaire	+ +	+	—	—	—	—	+	—	—	+	+	+	—	—	—	—	+	+	+	+	+	—	—	—	—	+	+	+

TABLE 2.

Control of O-antigens of *S. bilthoven*, *S. wassenaar* and *S. bonaire*.

serum	antigens	
	S. bilthoven	*S. kaolack*
S. bilthoven	1 : 10.000	1 : 5000
S. bilthoven absorbed by *S. kaolack*	1 : 80	1 : 80
	S. wassenaar	*S. greenside*
S. wassenaar	1 : 2500	1 : 5000
S. wassenaar absorbed by *S. greenside*	1 : 160	< 1 : 20
	S. bonaire	*S. greenside*
S. bonaire	1 : 5000	1 : 5000
S. bonaire absorbed by *S. greenside*	1 : 80	< 1 : 20

TABLE 3.

Control of H-antigens of *S. bilthoven*, *S. wassenaar* and *S. bonaire*.

serum	antigens	
	S. bilthoven	*S. paratyphi* a
S. bilthoven	1 : 40.000	1 : 40.000
S. bilthoven absorbed by *S. paratyphi* a	1 : 40	< 1 : 20
	S. wassenaar	*S. dublin*
S. wassenaar	1 : 10.000	1 : 5000
S. wassenaar absorbed by *S. dublin*	1 : 160	< 1 : 20
	S. bonaire	*S. tallahassee*
S. bonaire	1 : 40.000	1 : 40.000
S. bonaire absorbed by *S. tallahassee*	< 1 : 20	< 1 : 20

with all known *Salmonella-* and *Arizona*-antigens, except with *S. wassenaar* 1 : 80.

Both *S. wassenaar* and *S. bonaire* belong to *Salmonella* O-group 50, but apparently the O-antigens of *S. wassenaar* and *S. bonaire* are not identical with that of *S. greenside.* In Table 4 results of cross-absorption between *S. greenside*, *S. bonaire* and *S. wassenaar* are given.

After absorption of H-serum *S. wassenaar* by *S. dublin* a rest titer of 1 : 160 remained. No agglutination was seen with all known

TABLE 4.

The relationship of the O-antigens of *S. greenside*, *S. wassenaar* and *S. bonaire*.

O-sera	absorbed by	titers of O-antigen suspensions	
S. greenside	*S. bonaire*	*S. greenside*	1 : 160
		S. bonaire	< 1 : 20
		S. wassenaar	< 1 : 20
S. greenside	*S. wassenaar*	*S. greenside*	1 : 80
		S. bonaire	< 1 : 20
		S. wassenaar	< 1 : 20
S. bonaire	*S. greenside*	*S. bonaire*	1 : 80
		S. wassenaar	1 : 80
		S. greenside	< 1 : 20
S. wassenaar	*S. greenside*	*S. wassenaar*	1 : 80
		S. bonaire	1 : 80
		S. greenside	< 1 : 20

Salmonella and *Arizona* H-antigens, except with *Arizona* H13, 14 1 : 40 and *Arizona* H13, 15 1 : 80.

Absorption of the same serum by two other *S. dublin* strains gave the same result.

S. tallahassee was agglutinated to titer by and removed all H-agglutinins from *S. bonaire* H-serum. Therefore the antigenic formula of *S. wassenaar* is 50 : gp : — and of *S. bonaire* 50 : z_4z_{32} : —.

We are indebted to Prof. Dr. F. Kauffmann, Copenhagen and to Dr. P. R. Edwards, Atlanta for confirmation of our findings.

Summary.

Two new *Salmonella* types, *S. bilthoven*, 47ac : a : — and *S. wassenaar*, 50 : gp : — were isolated from reptiles in the Netherlands.

A new *Salmonella* type, *S. bonaire*, 50 : z_4z_{32} : —, was isolated from a cow on the Island Bonaire.

(From the National Institute of Public Health, Utrecht, the Netherlands).

SIX NEW *SALMONELLA* TYPES, ISOLATED IN GHANA (*S. VOLTA*, *S. AGONA*, *S. WA*, *S. TECHIMANI*, *S. MAMPONG* AND *S. TAFO*)

by

P. A. M. GUINÉE[1], **E. H. KAMPELMACHER**[2]
and **H. M. C. C. WILLEMS**[3]

(Received September 12, 1961).

All new types to be described were isolated by D. ZWART, Kumasi, College of Technology, Ghana.

S. volta was isolated from a swine, *S. agona*, *S. wa*, *S. techimani* and *S. tafo* from cattle and *S. mampong* from a lizzard (*Agama agama*). All strains showed the typical biochemical behaviour of the genus *Salmonella*. In table 1 the biochemical data are shown. In tables 2 and 3 the serological data for the six new *Salmonella* types are given.

Therefore the antigenic formulas of the six new types are as follows:

S.volta	11 : r : 1, z_{13}, z_{28}
S.agona	4, 12 : fgs : —
S.wa	16 : b : 1,5
S.techimani	28 : c : z_6
S.mampong	13, 22 : z_{35} : 1,6
S.tafo	1, 4, 12, 27 : z_{35} : 1,7

We are indebted to Prof. Dr. F. KAUFFMANN for confirmation of our findings.

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Biochemical behaviour of *S. volta, S. ag*

	glucose + gas	mannitol	adonitol	dulcitol	inositol	lactose	salicine	sucrose	indol	T.S.I.	ammoniumcitrate	methylred
S. volta	++	+	—	+	+	—	—	—	—	+	+	+
S. agona	++	+	—	+	+	—	—	—	—	+	+	+
S. wa	++	+	—	+	+	—	—	—	—	+	+	+
S. techimani	++	+	—	+	+	—	—	—	—	+	+	+
S. mampong	++	+	—	+	—	—	—	—	—	+	+	+
S. tafo	++	+	—	+	—	—	—	—	—	+	+	+

Summary.

Six new *Salmonella* types isolated in Ghana are described. *S.volta*, 11 : r : 1,z_{13}, z_{28} was isolated from a swine; *S.agona* 4,12 : fgs : —, *S.wa*, 16 : b : 1,5, *S.techimani*, 28 : c : z_6 and *S.tafo*, 1,4 12, 27 : z_{35} : 1,7 were isolated from cattle; *S.mampong*, 13,22 : z_{35} : 1,6, was isolated from a lizzard.

a, *S. techimani*, *S. mampong* and *S. tafo*.

phenyl-alanine	malonate	lysine decarboxylase	KCN	arabinose	rhamnose	trehalose	xylose	glycerol (Stern)	d-tartrate	l-tartrate	i-tartrate	citrate	mucate	gelatin
—	—	+	—	+	+	+	+	+	+	+	—30	+	+	—30
—	—	+	—	+	+	+	+	+	+	+	—30	+	+	+ 6
—	—	+	—	+	+	+	+	+	+	+	—30	+	+	—30
—	—	+	—	+	+	+	+	+	+	+	—30	+	+	—30
—	—	+	—	+	—	+	+	+	+	+	—30	+	+	—30
—	—	+	—	+	—	+	+	+	+	+	—30	+	+	—30

TABLE 2.

Control of O-antigens of *S. volta*, *S. agona*, *S. wa*, *S. techimani*, *S. mampong* and *S. tafo*.

serum	antigens	
	S. volta	*S. aberdeen*
S. volta	1 : 5000	1 : 5000
S. volta absorbed with *S. aberdeen*	< 1 : 20	< 1 : 20
	S. agona	*S. reading*
S. agona	1 : 1280	1 : 1280
S. agona absorbed with *S. reading*	< 1 : 20	< 1 : 20
	S. wa	*S. gaminara*
S. wa	1 : 2500	1 : 2500
S. wa absorbed with *S. gaminara*	< 1 : 20	< 1 : 20
	S. techimani	*S. tel-aviv*
S. techimani	1 : 1280	1 : 1280
S. techimani absorbed with *S. tel-aviv*	< 1 : 20	< 1 : 20
	S. mampong	*S. poona*
S. mampong	1 : 1280	1 : 1280
S. mampong absorbed with *S. poona*	< 1 : 20	< 1 : 20
	S. tafo	*S. abortus bovis*
S. tafo	1 : 640	1 : 640
S. tafo absorbed with *S. abortus bovis*	< 1 : 20	< 1 : 20

TABLE 3.

Control of H-antigens of *S. volta*, *S. agona*, *S. wa*, *S. techimani*, *S. mampong* and *S. tafo*.

serum	antigens			
	S. volta	*S. infantis*	*S. uganda*	*S. javiana*
S. volta	1 : 10000	1 : 1280	1 : 5000	1 : 10000
S. volta absorbed with *S infantis*	1 : 640	< 1 : 20	1 : 640	1 : 640
S. volta absorbed with *S. uganda* + *S. infantis*	1 : 32	[1])	< 1 : 2	1 : 8
S. volta absorbed with *S. infantis* + *S. uganda* + *S. javiana*	< 1 : 2	[1])	< 1 : 2	< 1 : 2
	S. agona	*S. derby*		*S. montevideo*
S. agona	1 : 10000	1 : 320		1 : 10000
S. agona absorbed with *S. derby*	1 : 640	< 1 : 20		1 : 640
S. agona absorbed with *S. derby* + *S. montevideo*	< 1 : 20	[1])		< 1 : 20
	S. wa	*S. minnesota* (*b*)		*S. cholerae suis* var. *kunz*
S. wa	1 : 20000	1 : 1280		1 : 20000
S. wa absorbed with *S. minnesota*	1 : 1280	< 1 : 20		1 : 1280
S. wa absorbed with *S. minnesota* + *S. cholerae suis* var. *kunz.*	1 : 20	[1])		1 : 20
	S. techimani	*S. yolo* (*c*)		*S. kentucky* (z6)
S. techimani	1 : 10000	1 : 2500		1 : 5000
S. techimani absorbed with *S. yolo*	1 : 320	< 1 : 20		1 : 320
S. techimani absorbed with *S. yolo* + *S. kentucky*	< 1 : 20	[1])		< 1 : 20
	S. mampong	*S. chittagong* (z35)		*S. inverness* (1,6)
S. mampong	1 : 10000	1 : 640		1 : 2500
S. mampong absorbed with *S. chittagong*	1 : 160	< 1 : 20		1 : 80
S. mampong absorbed with *S. chittagong* + + *S. inverness*	1 : 20	[1])		< 1 : 20
	S. tafo	*S. chittagong* (z35)		*S. gaminara* (1,7)
S. tafo	1 : 10000	1 : 320		1 : 10000
S. tafo absorbed with *S. chittagong*	1 : 1280	< 1 : 20		1 : 640
S. tafo absorbed with *S. chittagong* + *S. gaminara*	< 1 : 20	[1])		< 1 : 20

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[1]) not done

PERIODICAL